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THE BIOCHEMICAL JOURNAL

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A detailed list of *Suggestions to Authors, Abbreviations and Symbols, and Notes on Usage and Conventions* applicable to the *Biochemical Journal* was published in August 1953 (*Biochem. J.* 1953, 55, 1). *Notes on Preparation of Illustrations* for the *Biochemical Journal* was published in January 1956 (*Biochem. J.* 1956, 62). Copies of these pamphlets may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute, Chelsea Bridge Road, London, S.W. 1, price 1*s*. each post free.

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> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire).—(Abstract follows.)

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It would greatly facilitate the editors' task and thus be to the advantage of the author if, when submitting a paper which is part of a series, the author were to enclose reprints of the preceding immediately relevant parts.

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CORRIGENDA

The stabilization of purified human albumin to heat

By Margaret E. Mackay and N. H. Martin

Volume 65 (1957)

p. 285, col. 2, line 20

***for* with a 1 mm. *read* with a 1 cm.**

Phospholipids. 4. On the composition of hen's egg phospholipids

By D. N. Rhodes and C. H. Lea

Volume 65 (1957)

p. 526, date of receipt

for (*Received* 10 *August* 1954) read (*Received* 10 *August* 1956)

Enzymic Transfer of the Ribosyl Group from Inosine to Adenine*

By J. L. OTT and C. H. WERKMAN†
Department of Bacteriology, Iowa Agricultural Experiment Station and Industrial Science Research Institute, Iowa State College, Ames, Iowa, U.S.A.

(*Received* 9 *August* 1956)

The formation of adenosine from adenine and inosine was first described by Stephenson & Trim (1938). Washed cell suspensions of *Escherichia coli* were used, and the formation of ammonia from adenine was stimulated by catalytic amounts of inosine. Stephenson & Trim cautiously postulated a transamination from adenine to inosine as a possible mechanism for the reaction. MacNutt (1952), working with a dialysed extract from *Lactobacillus helveticus*, demonstrated the transfer of the deoxyribosyl group from one purine or pyrimidine nucleoside to another purine or pyrimidine base. Orthophosphate was not required, nor was deoxyribose 1-phosphate involved in the reaction. The mechanism postulated was a direct transfer with the enzyme named trans-*N*-glycosidase. In the case of a transfer between hypoxanthine deoxyriboside and adenine, the same results would be obtained from transamination as from trans-*N*-glycosidation. The trans-*N*-glycosidase action was confirmed by experiments with radioactive adenine (Kalckar, MacNutt & Hoff-Jørgensen, 1952). Transamination was shown to play no part in the reaction.

Hoffmann (1952) described similar transfer reactions catalysed by extracts of *E. coli*. In these experiments the trans-*N*-glycosidase action occurred only between purines and purine nucleosides, or pyrimidines and pyrimidine nucleosides. Deoxyribose 1-phosphate was a necessary intermediate in a transfer from purine nucleoside to pyrimidine base, or from pyrimidine nucleoside to purine base.

It has recently been shown that cell-free extracts from *E. coli* catalyse the formation of adenosine and hypoxanthine from adenine and inosine (Ott & Werkman, 1954, 1955). The enzyme preparations contain nucleoside phosphorylase active on adenosine and inosine. Coupled nucleoside phosphorylase reactions were postulated as a mechanism for the reaction (Ott & Werkman, 1957)

adenine + inosine ⇌ adenosine + hypoxanthine.

The catalysis of the reaction by cell-free extracts in the absence of added orthophosphate indicates that trans-*N*-ribosidation or transamination plays a role in the mechanism of the reaction (Ott & Werkman, 1957).

The purpose of the present paper is to establish the presence or absence of transamination as a reaction mechanism in the formation of adenosine and hypoxanthine from adenine and inosine.

METHODS

Methods for the preparation of cell-free extracts have been previously described (Ott & Werkman, 1954). These extracts may be stored at −10° for at least a year with no appreciable decrease in activity.

All chemicals were commercial preparations. Adenine labelled with ^{14}C in C-8 (1·09 μc/mg.) was obtained from Schwarz Laboratories Inc., Mount Vernon, New York. Chromatographically the radioactive adenine was identical with non-radioactive samples of adenine. Direct counting of the spots from chromatograms showed that all of the radioactivity placed at the origin moved to the adenine location. Radioautography of the compound showed only one active spot that corresponded to adenine. Absorption in ultraviolet light corresponded to the adenine-absorption curve. The purity of all other purine and nucleoside preparations was verified by spectrophotometry and paper chromatography.

The reaction was conducted in 10 ml. Erlenmeyer flasks at 37° in a Dubnoff metabolic shaking incubator. Sodium pyrophosphate buffer, pH 6·5 (90 μmoles), [8-^{14}C]adenine (1·4 μmoles) and inosine (1·5 μmoles) or adenosine (1·8 μmoles), in a total volume of 0·8 ml., were incubated for 30 min. The reaction was initiated by the addition of 0·1 ml. of cell-free extract diluted with pyrophosphate buffer to contain 0·8 mg. of protein. The reaction was stopped by the addition of 0·1 ml. of 1·5 N-H_2SO_4.

Quantitative determination of the products was made on portions of the reaction mixture after removal of the protein precipitate by centrifuging. The products were separated by paper chromatography on Whatman no. 1 paper. Portions of the supernatant fluid were placed at the origin and neutralized on the paper with NH_3 soln. Development was carried out at 7° by the ascending method in water adjusted to pH 10 with NH_3 soln. (Levenbook, 1955). After drying at 100° for 10 min., the ultraviolet-light-absorbing spots were located with a Model SL Mineralight equipped with a 2537 Å filter. The purines and nucleosides were quantitatively determined by ultraviolet spectrophotometry of the eluted spots according to Vischer & Chargaff (1948). Elution was carried out overnight in N-HCl at room temperature. A Beckman DU spectrophotometer with 1·0 cm. cells was used for all determinations. To compensate for possible ultraviolet-light-absorbing materials in the filter paper, the

* Journal paper No. J-3001 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 746.

† Supported in part by a grant from the U.S. Public Health Service.

concentration was determined from the density at the absorption maximum less the density at 290 mμ., as recommended by Vischer & Chargaff (1948).

Determinations of radioactivity were made on the compounds separated by paper chromatography. The spots that were visible under ultraviolet light were cut out and counted with a 1·4 mg./cm.^{2} end-window Geiger–Müller tube. All counts were made on paper.

Radioautography was carried out by exposing the developed chromatogram to Kodak No-Screen X-ray Safety Film for 3 days. The exposed film was developed according to the recommendations of the manufacturer.

Protein content of the extracts was determined by the biuret method of Weichselbaum (1946).

EXPERIMENTAL AND RESULTS

The formation of adenosine and hypoxanthine from adenine and inosine was carried out for 30 min. with [8-^{14}C]adenine as one substrate. The analyses of the reaction mixtures before and after incubation are shown in Table 1. Flasks 1–6 inclusive served as control flasks for substrate concentrations at zero time. Acid was added to these flasks before the enzyme preparation. No exchange of radioactivity occurred between adenine and inosine (flask 5) or between adenine and adenosine (flask 6) in the presence of enzyme inactivated by acid. The incubated flask containing adenine (flask 8) showed no decrease in concentration after incubation with the enzyme. Some decrease in concentration of inosine (flask 9) and adenosine (flask 10) occurred. This decrease was due to nucleoside phosphorylase activity of the cell-free extract with formation of the purine bases and ribose 1-phosphate (Ott & Werkman, 1957). The flask that contained [8-^{14}C]-adenine and inosine as substrate (flask 11) showed the formation of adenosine and hypoxanthine. Adenosine and adenine were the only radioactive compounds, with adenosine having essentially the same specific activity (13 800 counts/min./μmole) as the adenine (14 330 counts/min./μmole). The sum of the radioactivity found in the two compounds, 20 900 counts/min., corresponds satisfactorily with the activity in the corresponding flask at zero time, i.e. flask 5 with 20 960 counts/min. This complete recovery of radioactivity indicates that there was no other radioactive compound formed from adenine. A comparison of flask 11 with flask 5 shows that there is essentially a 1:1 ratio of reactants disappearing to products formed. This stoicheiometry agrees with the results obtained when the reaction was carried out with non-radioactive compounds (Ott & Werkman, 1957).

An exchange of radioactivity occurs between adenine and adenosine in the presence of enzyme (flask 12). Essentially no change in concentration occurred during the incubation. Radioactive equilibrium was not reached in 30 min. At equilibrium the specific activity of each should be one-half of the specific activity of the original adenine or approximately 7000 counts/min./μmole.

Table 1. *Formation of radioactive adenosine from* [8-^{14}C]*adenine and inosine*

Indicated substrates were added to a test system which contained 0·1 ml. of cell-free extract (0·8 mg. of protein) and 90 μmoles of pyrophosphate buffer (pH 6·5). Total volume, 0·9 ml.; incubated for 30 min. at 37°. Protein was precipitated with 0·1 ml. of 1·5N-H_2SO_4.

Flask no.	Substrate	Time of reaction (min.)	Compound determined	Radio-activity (counts/min.)	Quantity found (μmoles)	Change in quantity (μmole)	Specific activity (counts/min./μmole)
1	None	0	None	—	—	—	—
2	Adenine	0	Adenine	20 500	1·4	—	14 500
3	Inosine	0	Inosine	—	1·5	—	—
4	Adenosine	0	Adenosine	—	1·8	—	—
5	Adenine and inosine	0	Adenine	20 960	1·4	—	14 300
			Inosine	—	1·5	—	—
6	Adenine and adenosine	0	Adenine	19 700	1·4	—	14 000
			Adenosine	—	1·8	—	—
7	None	30	None	—	—	—	—
8	Adenine	30	Adenine	19 700	1·4	0·0	14 200
9	Inosine	30	Inosine	—	1·3	−0·2	—
10	Adenosine	30	Adenosine	—	1·6	−0·2	—
11	Adenine and inosine	30	Adenine	11 780	0·8	−0·6	14 350
			Adenosine	9 120	0·7	+0·7	13 800
			Hypoxanthine	0	0·6	+0·6	—
			Inosine	0	0·8	−0·7	—
12	Adenine and adenosine	30	Adenine	11 240	1·5	+0·1	7 750
			Adenosine	8 980	1·7	−0·1	5 210

The formation of radioactive adenosine from [8-^{14}C]adenine and inosine is shown by the radioautograph reproduced in Fig. 1. With adenine alone, no other radioactive spot was detectable. In the presence of inosine and enzyme, [8-^{14}C]adenine formed radioactive adenosine. The spots corresponding to hypoxanthine and inosine were devoid of activity. The exchange of radioactivity between adenine and adenosine is also shown on the radioautograph.

DISCUSSION

The formation of radioactive adenosine from [8-^{14}C]adenine and inosine establishes the mechanism of the reaction as a transfer of the ribosyl group. If the amino group had been transferred from adenine to inosine, the hypoxanthine formed would have been radioactive. Only by a transfer of the ribosyl group would radioactivity be found in adenosine. The absence of radioactivity in the hypoxanthine formed during the reaction rules out a transamination reaction. The essentially undiluted specific activity of the adenosine indicates that it was formed only from adenine and that there were few, if any, side reactions. The 1:1 ratio of reactants disappearing to products formed further indicates the absence of side reactions. Although the cell-free extract shows nucleoside phosphorylase activity on inosine and adenosine, the rates of the two reactions are such that there is no net synthesis of ribose 1-phosphate when both adenine and inosine or adenosine and hypoxanthine are incubated together (Ott & Werkman, 1957).

The transfer of the ribosyl group was demonstrated from inosine to adenine and from adenosine to adenine. Since the reaction is reversible (Ott & Werkman, 1957), it is likely that the ribosyl group can be transferred from adenosine to hypoxanthine.

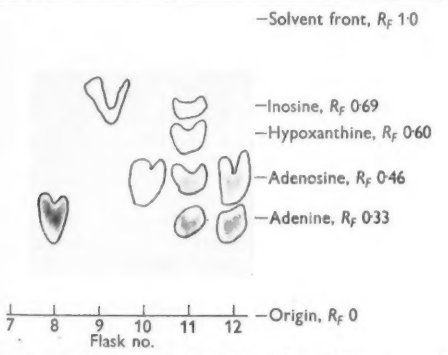

Fig. 1. Radioautograph of the formation of adenosine and hypoxanthine from [8-^{14}C]adenine and inosine. Test system and conditions were the same as in Table 1. Spots shown in outline were those visible under ultraviolet light. Film was exposed to the chromatogram for 3 days.

The ribosyl transfer is similar to the deoxyribosyl transfer in *L. helveticus* with the analogous deoxyribose compounds as described by Kalckar *et al.* (1952). In both cases transamination has been eliminated as a reaction mechanism. The mechanism with the deoxyribosyl compounds and the *L. helveticus* preparations was shown to be a direct transfer with no involvement of deoxyribose 1-phosphate (MacNutt, 1952). The nucleoside phosphorylase activity of the *E. coli* extracts and the reactions of the purine bases with ribose 1-phosphate to form the nucleosides (Ott & Werkman, 1957) indicate that the reaction mechanism in *E. coli* involves a ribosyl transfer with ribose 1-phosphate as the intermediate. The possibility of a direct transfer of the ribosyl group with *E. coli* extracts has not been eliminated. The data obtained (Ott & Werkman, 1957) indicate that the reaction is the result of coupled nucleoside phosphorylase reactions. Enzyme preparations from *E. coli* that catalyse the reaction in the complete absence of orthophosphate or that are devoid of nucleoside phosphorylase activity have not been obtained. The amount of orthophosphate present in the extract was 0·16 μmole (cf. Ott & Werkman, 1957, for discussion).

SUMMARY

1. The formation of radioactive adenosine from [8-^{14}C]adenine and inosine catalysed by cell-free extracts from *Escherichia coli* establishes the transfer of the ribosyl group as a mechanism of the reaction. Hypoxanthine formed during the reaction was devoid of radioactivity, a result that eliminates transamination as a mechanism.

2. The involvement of ribose 1-phosphate as an intermediate in the reaction and the relationship of the reactions to the deoxyribosyl transfer in *Lactobacillus helveticus* preparations is discussed.

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The Distribution of Guanidines, Phosphagens and *N*-Amidino Phosphokinases in Echinoids

By D. E. GRIFFITHS,* J. F. MORRISON and A. H. ENNOR
Department of Biochemistry, John Curtin School of Medical Research, The Australian National University, Canberra, A.C.T., Australia

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The class Echinoidea assumed prominence in the field of comparative biochemistry when it was shown by Needham, Needham, Baldwin & Yudkin (1932) that the jaw muscles of *Strongylocentrotus lividus* contained both phosphocreatine and phosphoarginine. Further work by Baldwin & Needham (1937) showed that extracts of the jaw muscles of two other echinoids, *Paracentrotus lividus* and *Sphaerechinus granularis*, were capable of phosphorylating both creatine and arginine. This result suggested that the muscles of both of these organisms contained phosphocreatine and phosphoarginine, although neither phosphagen was identified. Earlier Meyerhof (1930) had advanced the hypothesis that phosphoarginine was the characteristic phosphagen in the muscles of invertebrates and phosphocreatine was characteristic of the vertebrates. The occurrence of both phosphagens in the echinoids studied provided biochemical support (Needham *et al.* 1932) for the evolutionary theory of Bateson (1886), namely that the echinoderms were the immediate precursors of the primitive chordates. Later work has shown that the premises on which Meyerhof's hypothesis was based are not correct. It has been shown that phosphocreatine is not confined to the vertebrates and those animals intermediate between vertebrates and invertebrates, but that it is also present in sponges (Roche & Robin, 1954) and annelids (Hobson & Rees, 1955). Moreover, other phosphorylated guanidines, e.g. phosphotaurocyamine, phosphoglycocyamine and phospholombricine, also occur in invertebrate muscle (Thoai, Roche, Robin & Thiem, 1953*a*; Thoai & Robin, 1954).

The earlier work on echinoids was concerned mainly with the study of the distribution of free guanidines. Arnold & Luck (1933) showed that arginine was present in the jaw muscles of *Strongylocentrotus franciscanus*, and Holtz & Thielmann (1924) found arginine in *Arbacia pustulosa*. The first positive identification of creatine in echinoids was made by Greenwald (1946), who isolated potassium creatinine picrate from the testes of *Strongylocentrotus* sp. Before this, creatine had been identified by the non-specific Jaffe reaction (cf. Myers, 1920).

The development of improved techniques for the identification and estimation of guanidine compounds and the availability of two Australian echinoids, *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* Valenciennes, prompted an investigation of the distribution of guanidines and phosphagens in these genera. At the same time a study was made of the distribution of the transphosphorylases involved in the metabolism of these compounds. The results of such a study are described here and indicate that *C. rodgersii* contains arginine, phosphoarginine and arginine phosphokinase only, whereas *H. erythrogramma* contains creatine, phosphocreatine and creatine phosphokinase, as well as arginine, phosphoarginine and arginine phosphokinase.

Abbreviations. The following abbreviations will be used: PA, phosphoarginine; APK, arginine phosphokinase; PC, phosphocreatine; CPK, creatine phosphokinase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

MATERIALS

Specimens of *C. rodgersii* and *H. erythrogramma* were collected in the Sydney area during the summer months. The gonads and jaw apparatus (Aristotle's lantern) were excised and frozen immediately by dropping them into liquid N_2. When the tissues had reached the temperature of liquid N_2, they were removed, placed in polythene bags and stored in solid CO_2 for transport to the laboratory. No distinction was made between male and female gonads.

Phosphagens. PA was isolated from the tail muscle of the sea-crayfish (*Jasus verreauxi*) as a barium salt by the method of Ennor, Morrison & Rosenberg (1956) and converted into the sodium salt as described by Morrison, Griffiths & Ennor (1957). Sodium PC was synthesized by the method of Ennor & Stocken (1948). Phosphotaurocyamine was isolated as the barium salt after phosphorylation of taurocyamine by $POCl_3$ (cf. Ennor & Stocken, 1948). Phosphoglycocyamine was prepared as described by Rosenberg (1955) and isolated as the sodium salt.

Guanidines. Arginine, glycocyamine and creatine were products of British Drug Houses Ltd.; the last two compounds were purified by recrystallization from water.

* Australian National University Research Scholar.

Taurocyamine (*N*-amidinotaurine) was prepared by treatment of taurine with *S*-ethylthiourea as described by Brand & Brand (1942); its purity was checked by elementary analysis.

Arginase. Arginase was prepared from ox liver by the method described by Greenberg (1951). The preparation was taken to stage F and freeze-dried. The enzyme was dissolved in 0·05 M *N*-ethylmorpholine buffer (pH 8·4) before use. Such solutions contained sufficient Mn^{2+} ions to activate the enzyme but insufficient to interfere with the colorimetric method for the estimation of guanidines.

Creatine phosphokinase. CPK was prepared as described by Ennor & Rosenberg (1954*a*). The freeze-dried enzyme was dissolved in 0·05 M *N*-ethylmorpholine buffer of the appropriate pH before use.

Arginine phosphokinase. APK was prepared by the method of Morrison *et al.* (1957) and stored as a 2·3% (w/v) solution in 50% (v/v) glycerol. Dilutions of the enzyme were made with 0·05 M *N*-ethylmorpholine buffer of the appropriate pH.

METHODS

Estimation and detection of guanidines. The guanidine content of tissue extracts was estimated by the colorimetric method of Rosenberg, Ennor & Morrison (1956). Guanidines were demonstrated on paper chromatograms by spraying with a 1:1 mixture of 5 N-NaOH and the α-naphthol–diacetyl reagent of Rosenberg *et al.* (1956). Monosubstituted guanidines were demonstrated also by the Sakaguchi reaction as described by Acher & Crocker (1952).

Estimation of protein. Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949), crystalline bovine-serum albumin being used as the standard.

Study of guanidines

Extraction of guanidines. The method used for the extraction of guanidines from tissues was essentially that of Thoai, Roche, Robin & Thiem (1953*b*). The frozen material was treated in a Waring Blendor with 2 vol. of water and the mixture then adjusted to pH 3 by the addition of 10 N-H_2SO_4. (Large amounts of CO_2 were evolved on the addition of acid to extracts of the jaw apparatus.) It was then boiled for 2 min., cooled, and the precipitate removed by centrifuging. Samples were withdrawn for the determination of the guanidine content and the remainder was treated with a saturated solution of basic lead acetate until there was no further precipitation, and the excess of lead was removed with H_2S. All the extracts obtained were clear, but were sometimes coloured and in these cases the colour was removed by treatment with a small amount of active charcoal. The extract was then freeze-dried and the residue dissolved in a small volume of water; insoluble material was removed by centrifuging and the supernatant examined enzymically and by paper chromatography.

Paper chromatography. Paper-chromatographic examination of the tissue extracts was carried out by the ascending method at room temperature (20–22°). Sheets of Whatman no. 3 MM paper (12 in. × 12 in.) were used as a better resolution of guanidines was obtained than with Whatman no. 1 paper.

Many solvent systems were investigated, including those recommended by Block & Zweig (1955) and Roche, Thoai & Hatt (1954). Development of tissue extracts in alkaline solvents led to excessive 'tailing' of the guanidine spots, and there was not sufficient resolution of guanidines in the acidic solvents used by these authors to enable positive identification. As will be seen below, extracts of the tissue of *H. erythrogramma* contain arginine and creatine, and excellent separation of these guanidines was obtained with *n*-butanol–6 N-HCl (70:30, v/v; Makisumi, 1952) and phenol–water (80:20, w/v) saturated with SO_2 (Crawford, 1951); see Table 1. The following solvents were used in conjunction with *n*-butanol–HCl and phenol–water–SO_2 even though the separation of guanidines was not as good: *n*-propanol–NH_3 soln. (sp.gr. 0·88)–water (60:30:10; Hanes & Isherwood, 1949); pyridine–*iso*pentanol–water (80:40:70; Roche *et al.* 1954); acetone–*n*-propanol–NH_3 soln. (2 N)–water (50:10:30:10), acetone–NH_3 soln. (2 N)–water (60:30:10) and *n*-propanol–dioxan–NH_3 soln. (2 N) (40:10:50).

The guanidines present in tissue extracts were further characterized by enzymic means as the presence of impurities made the preparation of crystalline derivatives difficult. Moreover, the enzymes used (arginase, CPK and APK) show such a high degree of specificity that they provide an excellent means for the characterization of arginine and creatine. Tissue extracts adjusted to pH 8·5 with N-NaOH were incubated with arginase in a system which contained 0·1 ml. of extract, 0·1 ml. of *N*-ethylmorpholine buffer (0·5 M, pH 8·5), 0·1 ml. of arginase (containing 0·1–1·0 mg. of protein) and 0·7 ml. of water. The mixture was incubated at 37° for 1–2 hr., after which 1·0 ml. of an EDTA–NaOH mixture containing 0·4 ml. of 0·2 M EDTA (adjusted to pH 7·6) and 0·6 ml. of 5 N-NaOH was added. The presence of arginine was detected by estimation of the total guanidine before and after incubation with arginase. By this means it was also possible to estimate the amount of arginine in the extract as it is almost completely destroyed by arginase. Appropriate control experiments were always carried out to ensure that the extracts contained no substances capable of inhibiting arginase activity and thus producing misleading results.

Arginine and creatine were also characterized by incubation with APK and CPK in a system which contained 0·1 ml. of extract (pH 8·5), 0·1 ml. of ATP (5×10^{-2} M), 0·1 ml. of $MgSO_4$ (10^{-2} M), 0·1 ml. of *N*-ethylmorpholine buffer (0·5 M, pH 8·5), 0·2 ml. of APK or CPK (containing 1 mg. of protein) and 0·4 ml. of water. The reaction mixture was incubated at 5° for 1–2 hr. and the guanidine disappearance measured after the addition of 1·0 ml. of the EDTA–NaOH mixture described above.

When chromatographic analysis of the guanidines present in tissue extracts indicated the presence of both creatine and arginine, enzymic identification of the guanidines was

Table 1. *R_F values of guanidine compounds*

Guanidines were chromatographed on Whatman no. 3 MM paper at room temperature (20–22°) by the ascending method.

	R_F values	
Guanidine	Butanol–6 N-HCl (70:30, v/v)	Phenol–water (80:20, w/v) saturated with SO
Creatine	0·52	0·67
Arginine	0·25	0·39
Glycocyamine	(trails)	0·50
Taurocyamine	0·46	0·73

carried out by colorimetric estimation after each of the following treatments: (1) incubation of samples of the extract with APK and CPK; (2) incubation of a sample of the extract with CPK after destruction of the arginine with arginase; (3) incubation of the separated guanidine with the appropriate *N*-amidino phosphokinase. For this purpose arginine and creatine were separated by passing the extract through a column of Zeo-Karb 216 (Na form) when creatine passed through. The column was washed with water and the adsorbed arginine eluted with 2N-NH_3 soln. The eluate was freeze-dried after removal of most of the ammonia by aeration. This method was used only for the extracts from the jaw muscles of *H. erythrogramma* which contained an unknown inhibitor of arginase.

Study of the phosphagens

Isolation of phosphagens. The jaw apparatus was extracted with 2 vol. of ice-cold 9% (w/v) TCA by treatment in a Waring Blendor for 2 min. The mixture was filtered on a Büchner funnel (Whatman no. 531 paper) and the filter cake re-extracted with 1 vol. of ice-cold 5% (w/v) TCA. After filtration the filtrates were combined, adjusted to pH 9 by the addition of 10N-NaOH and the resultant precipitate was removed by centrifuging. These operations were carried out in the cold room at 5°. The pH of the extract was then adjusted to 8·2 by the addition of 5N-HCl, excess of barium acetate (M) was added and the pH readjusted to 8·2. The insoluble barium salts were removed by centrifuging. To the supernatant was added 4 vol. of ethanol, the pH was adjusted to 8·2 and the precipitate was allowed to settle overnight. The precipitate was removed by centrifuging, washed with ethanol and ether and dried *in vacuo*.

The gonads were extracted as described above, but 1 vol. of ethanol was added to the TCA extract before neutralization in order to precipitate the glycogen. Further fractionation was carried out as described by LePage (1951).

The water-soluble, ethanol-insoluble barium salts containing the phosphagen were suspended in a small volume of water and sufficient 5N-HCl to effect solution was added. The solution was immediately adjusted to pH 9 by the addition of 5N-NaOH. The inorganic phosphate so precipitated was removed by centrifuging and discarded; 4 vol. of ethanol was added to the supernatant and the barium salt of the phosphagen collected by centrifuging, washed with ethanol and ether and dried *in vacuo*.

Preparation of the sodium salts of the phosphagens. Conversion of the barium salts into sodium salts by the addition of Na_2SO_4 led to a marked loss of phosphagen when PC was present, so that an ion-exchange column was used from which 85% recoveries were obtained. Solutions of the barium salts were passed through a column of Zeo-Karb 225 (Na form). The effluent was freeze-dried and the residue dissolved in a small volume of water. In no case did the material so prepared contain guanidine other than that bound to P.

Paper chromatography. Paper chromatography of the tissue phosphagens in solvent systems used by other authors, e.g. propanol–NH_3 soln.–water (Hobson & Rees, 1955), pyridine–*iso*pentanol–water (Roche *et al.* 1954), proved unsatisfactory owing to the marked 'tailing' of the compounds. Moreover, there was such poor separation of marker phosphagens, PA, PC, phosphotaurocyamine and phosphoglycocyamine, that these solvents could not be relied on for use in positive identification of unknown phosphagens. Attempts were made to find solvents which would give good separation of the various phosphagens, but they were unsuccessful. The phosphagens were therefore identified by chromatography of the free bases after acid hydrolysis. The barium salts of the phosphagens were dissolved in N-HCl and allowed to hydrolyse by standing at room temperature for 24 hr. Alternatively the solutions of the sodium salts of the phosphagens were spotted on the paper and hydrolysed by allowing the paper to remain for 1 hr. in a jar filled with HCl vapour before development with solvent. Both procedures were effective in hydrolysing the phosphagens of echinoid tissues. The conditions for chromatography were the same as those described previously.

Enzymic characterization of phosphagens. Solutions of the sodium salts of the phosphagens were incubated separately with APK and CPK (1 mg. of protein/ml.) for 1 hr. at 5° under the conditions described for the determination of APK activity (Morrison *et al.* 1957). The release of arginine or creatine by the respective phosphokinases (which contained no phosphatase or phosphoamidase activity) in the presence of ADP was taken as evidence for the presence of the relevant phosphagen.

Study of the N-*amidino phosphokinases*

The jaw apparatus only of both echinoids was examined for the presence of *N*-amidino phosphokinases. The frozen jaw apparatus was passed twice through a mincer and extracted with 2 vol. of water by stirring mechanically for 30 min. The mixture was centrifuged for 15 min., the residue re-extracted with 1 vol. of water for 30 min., centrifuged and the supernatants were combined. The extract was then fractionated with solid $(NH_4)_2SO_4$. All operations were carried out at 5°. APK and CPK activities were determined under the same conditions as described by Morrison *et al.* (1957) for the determination of APK activity. In each case, the appropriate controls for phosphoamidase activity were set up.

RESULTS

The results of the investigation into the nature of the guanidines present in extracts of the jaw muscles and gonads of *C. rodgersii* and *H. erythrogramma* are shown in Table 2.

It may be seen that arginine only is present in the jaw muscles and gonads of *C. rodgersii* and that both arginine and creatine are present in similar tissues of *H. erythrogramma*. Also the guanidine content of the gonads of both echinoids is much greater than that of the jaw muscles, which is the reverse of the findings in vertebrate tissue (cf. Ennor & Rosenberg, 1952). The proportion of the total guanidine in *H. erythrogramma* which is represented by arginine is much less in the jaw muscles than in the gonads.

A study of the phosphagens present (Table 3) shows that only PA is present in the jaw muscles and gonads of *C. rodgersii*, whereas these tissues in *H. erythrogramma* contain both PA and PC. No detailed investigations were carried out to determine the relative amounts of each of the phosphorylated guanidines in the tissues of *H. erythrogramma*, but qualitative analyses indicated that

the ratio PA:PC was about the same as that of the free guanidines.

Examination of aqueous extracts of the jaw muscles of *C. rodgersii* showed that only APK was present. Fractionation of the extract showed that the major portion of the enzyme was precipitated between the addition of 42 and 56 g. of $(NH_4)_2SO_4$/100 ml. No CPK activity was detected in the original extract or in any of the fractions obtained as a result of $(NH_4)_2SO_4$ fractionation. The APK isolated did not phosphorylate creatine, glycocyamine or taurocyamine. The enzyme is similar to that isolated from crayfish muscle (Morrison *et al.* 1957) in that it is activated by Mg^{2+} and Mn^{2+} ions, but not by Ca^{2+} ions.

Aqueous extracts of the jaw muscles of *H. erythrogramma* contained both APK and CPK, which could be separated by $(NH_4)_2SO_4$ fractionation. Table 4 shows that CPK is precipitated between the addition of 28 and 35 g. of $(NH_4)_2SO_4$/100 ml., whereas APK is precipitated between the addition of 42 and 63 g. of $(NH_4)_2SO_4$/100 ml. It is also clear in *H. erythrogramma* that the total units of activity of APK and CPK are approximately equal. The APK isolated was activated by Mg^{2+} and Mn^{2+} ions, but not by Ca^{2+} ions, and in this respect is

Table 2. *Distribution of guanidines in the jaw muscles and gonads of* C. rodgersii *and* H. erythrogramma

Methods used for the identification of guanidines have been described in the text.

Echinoid	Tissue	Guanidine content (mg./100 g.) Creatine	Arginine	Phosphorylation by CPK (%)	Phosphorylation by APK (%)
Centrostephanus rodgersii	Jaw muscles	0	82·5	—	60
	Gonads	0	250	0	80
Heliocidaris erythrogramma	Jaw muscles	27	10	24*	36*
	Gonads	77	124	30†	—

* Tests carried out on arginine and creatine separated by passing through a column of Zeo-Karb 216 (Na form).
† Test carried out on creatine remaining in extract after destruction of arginine with arginase.

Table 3. *Distribution of phosphagens in the jaw muscles and gonads of* C. rodgersii *and* H. erythrogramma

Methods used for the identification of phosphagens have been described in the text. Abbreviations: PA, phosphoarginine; PC, phosphocreatinine; APK, arginine phosphokinase; CPK, creatine phosphokinase.

Echinoid	Tissue	Phosphagens present	Guanidine liberated by APK	CPK
Centrostephanus rodgersii	Jaw muscles	PA	+	0
	Gonads	PA	+	0
Heliocidaris erythrogramma	Jaw muscles	PA and PC	+	+
	Gonads	PA and PC	+	+

Table 4. *Arginine phosphokinase (APK) and creatine phosphokinase (CPK) activity of fractions isolated by ammonium sulphate fractionation of extracts* (100 *ml.*) *of the jaw apparatus of* C. rodgersii *and* H. erythrogramma

A unit of enzyme activity is defined as the amount of enzyme which releases 1 μmole of creatine or arginine in 1 min. at pH 7·2 and 5°. Specific activity (S.A.) is defined as the number of units of enzyme activity/mg. of protein. Fraction 1 was obtained by the addition of 21 g. of $(NH_4)_2SO_4$ to the extract. Fractions 2–5 inclusive were obtained by the stepwise addition of 7 g. of $(NH_4)_2SO_4$ to the supernatant from the preceding fraction. Fraction 6 was obtained by the addition of 14 g. of $(NH_4)_2SO_4$ to fraction 5 (or fraction 4 in *C. rodgersii*).

Fraction	*C. rodgersii* APK 10^2 S.A.	*H. erythrogramma* APK 10^2 S.A.	APK Total units	CPK 10^2 S.A.	CPK Total units
Original	230	12	56·3	8·3	39·0
1	68	1·7	3·2	3·1	5·6
2	49	1·4	0·97	11·4	7·9
3	26	1·2	0·68	41	23·0
4	244	10·4	6·45	5	3·1
5	—	74	11·8	0·6	0·1
6	825	81	10·5	0	0

similar to APK isolated from crayfish muscle and from *C. rodgersii*. The CPK, on the other hand, was activated by all three cations and is thus similar to CPK isolated from rabbit muscle (Rosenberg & Ennor, 1955; Kuby, Noda & Lardy, 1954). A comparison of the activation of the two enzymes by Mg^{2+}, Mn^{2+} and Ca^{2+} ions is shown in Fig. 1.

DISCUSSION

The present work has clearly established that the echinoid *Centrostephanus rodgersii* contains only one phosphagen (PA) in the jaw muscles and thus provides the second example of a mono-phosphagen type in this group. The first example was described by Baldwin & Yudkin (1950), who found only PA in *Arbacia punctulata*. The general assumption (cf. Baldwin & Needham, 1937; Wald, 1952; Florkin, 1949) that both PA and PC are found in echinoids is thus disproved and the results emphasize the necessity for examining a number of species within a class before concluding that a particular phosphagen(s) is characteristic of that class.

The occurrence of both PA and PC in certain echinoids and only PA in others suggests that some members of this group may contain only PC, but no such echinoid has been described. The investigations up to the present have covered an insufficient number of echinoids to demonstrate with certainty any pattern so far as the occurrence of phosphagens is concerned. Nevertheless, it is of interest to note that those echinoids whose jaw muscles have been shown to contain both PA and PC [*Paracentrotus lividus*, *Sphaerechinus granularis* (Baldwin & Needham, 1937); *Strongylocentrotus lividus*, *Echinus esculentus* (Baldwin & Yudkin, 1950; *Heliocidaris erythrogramma* (present work)] all belong to the order Camarodonta (Hyman, 1955*a*). Conversely, members of other orders, e.g. Aulodonta (*Centrostephanus rodgersii*) and Stirodonta (*Arbacia punctulata*), contain only PA. No members of the other orders have been investigated.

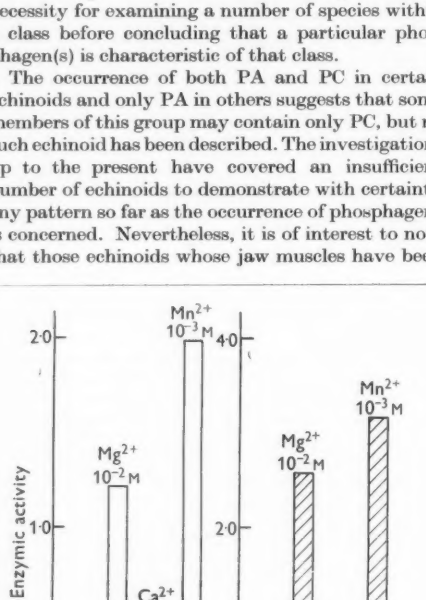

Fig. 1. Activation by bivalent metals of arginine phosphokinase (APK) and creatine phosphokinase (CPK) fractions from the jaw muscles of *H. erythrogramma*. Fractions were isolated and activity determinations carried out in the forward reaction as described in the text. □, CPK fraction; ▨, APK fraction.

The only free guanidines which were detected in the present work were arginine and creatine, although it is possible that others were present at concentrations less than 1% of the total guanidine. Thus it might have been expected that *H. erythrogramma*, which contains PC, would also contain trace amounts of glycocyamine in view of the results of Roche, Thoai, Robin, Garcia & Hatt (1952) and Thoai & Robin (1951), which indicated the presence of glycocyamine and the enzyme methylating glycocyamine in *Paracentrotus lividus* and *Sphaerechinus granularis*.

Although Baldwin & Needham (1937) showed that extracts of the muscles of *P. lividus* and *S. granularis* were able to phosphorylate both creatine and arginine, their results did not indicate whether this phosphorylation was carried out by one or two enzymes. The present work clearly establishes the presence of two enzymes of approximately equal activities in *H. erythrogramma*. Moreover, the general properties of the APK and CPK present are similar to those of APK isolated from crayfish muscle (Morrison *et al.* 1957) and CPK isolated from rabbit muscle (Rosenberg & Ennor, 1955) and from *Pyura stolonifera* (Griffiths, Morrison & Ennor, unpublished observations). The echinoids possess seven different groups of muscle in their jaw apparatus (Hyman, 1955*b*), and it has been shown by Needham *et al.* (1932) that both the protractor and retractor muscles of *Strongylocentrotus lividus* contain PA and PC. It would seem therefore that in this species and in *H. erythrogramma* both phosphagens are present in the same muscles, and it follows that the free guanidines and the transphosphorylation enzymes are also present.

There would seem little point, in view of the present state of knowledge, in speculating on the possible evolutionary reasons for the appearance of the two phosphagens in the jaw muscles of *H. erythrogramma*. It is difficult to picture the functional interrelationship which might exist between the two phosphagens, for although the role of PA in invertebrate muscle appears to be similar to that of PC in vertebrate muscle it is hard to conceive of the two phosphagens both serving as 'reservoirs' for the adenylic acid system in the same muscle. However, in the absence of evidence to the contrary, it must be assumed that such is the role of

the two phosphagens, although the possibility (Ennor & Rosenberg, 1952) that one or other of these phosphagens 'may contribute to endergonic reactions directly and without the mediation of the latter (adenylic acid) system' must be borne in mind. It is not clear how the function of each phosphagen may be determined. Ennor & Rosenberg (1954*b*) have shown that the incorporation of ^{32}P into PC in rabbit muscle is extremely rapid, and it is likely that this would also occur with PA and PC in echinoid muscle; it would thus be difficult to detect any differential incorporation of ^{32}P into the two phosphagens, so that the application of ^{32}P to the problem would seem of little value.

SUMMARY

1. Two echinoids, *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* Valenciennes, have been examined for the presence of free guanidine, their phosphorylated derivatives and their respective *N*-amidino phosphokinases.

2. *C. rodgersii* has been shown to contain arginine, phosphoarginine and arginine phosphokinase. *H. erythrogramma*, on the other hand, contained both creatine and arginine together with their phosphorylated derivatives and *N*-amidino phosphokinases.

3. The creatine and arginine phosphokinases in *H. erythrogramma* are readily separable and are similar in properties to those enzymes isolated from vertebrate and invertebrate sources.

4. Methods for the chromatographic and enzymic characterization of creatine and arginine are described.

We are indebted to Dr J. W. Evans, Director of the Australian Museum, Sydney, for the use of Museum facilities, and to Mrs J. W. Evans, Miss Elizabeth Pope and Miss P. McDonald of the Australian Museum for assistance in locating and collecting specimens. Our thanks are also due to Miss Pope for the identification of the specimens and to Mr D. Maguire and Miss B. Giddey for technical assistance.

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The Biosynthesis of Penicillin

5. COMPARISON OF VALINE AND HYDROXYVALINE AS PENICILLIN PRECURSORS*

By H. R. V. Arnstein and Margaret E. Clubb
National Institute for Medical Research, Mill Hill, London, N.W. 7

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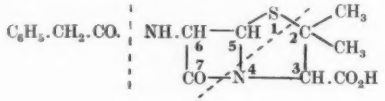
Both cystine (Arnstein & Grant, 1954*a*, *b*; Stevens, Vohra, Inamine & Roholt, 1953) and valine (Arnstein & Grant, 1954*a*; Stevens, Vohra & De Long, 1954) are utilized by *Penicillium chrysogenum* for penicillin biosynthesis, and it has been shown that L-cystine is incorporated intact, presumably after reduction to cysteine, as a precursor of the sulphur atom, the β-lactam carbon atoms ($C_{(6)}$–$C_{(7)}$) and the side-chain nitrogen atom of benzylpenicillin (I) (Arnstein & Grant, 1954*b*). The incorporation of valine, however, was inferred only from experiments with ^{14}C-labelled DL-valine, and no direct utilization of the intact amino acid has been demonstrated.



(I)

In the present work, the biosynthesis of penicillin from valine has therefore been investigated in more detail with DL-valine labelled with both ^{14}C and ^{15}N. Penicillin biosynthesis has also been studied by incubating D- and L-[*carboxy*-^{14}C]valine with washed mycelium for 1–4 hr., as it seemed possible that differences in the utilization of the D- and L-enantiomorphs might be more easily demonstrated in such short-term experiments than in complete fermentations. In view of the D-configuration of the α-carbon atom of naturally occurring penicillamine it was expected that D-valine would be preferentially utilized. It was found, however, that both D- and L-valine were converted into penicillin to an equal extent, provided that allowance was made for differences in uptake of the two forms by the mycelial cells. Experiments are also described which exclude β-hydroxyvaline as an intermediate in the conversion of valine into penicillin. Some of this work has been briefly reported at the 341st meeting of the Biochemical Society (Arnstein & Clubb, 1955).

* Part 4: Halliday & Arnstein (1956).

EXPERIMENTAL

Fermentations

Organism and preparation of cultures. In the present work strains WIS 48–701 and WIS 51–20 of *Penicillium chrysogenum* were used, the latter giving somewhat better yields (up to 550 i.u./ml.) under our experimental conditions. Subcultures and spore inocula were prepared as described previously (Arnstein & Grant, 1954*b*).

Details of medium and fermentations. All fermentations were carried out in a stirred aerated jar fermenter containing 1–1·5 l. of synthetic medium under conditions identical with those used earlier (Arnstein & Grant, 1954*b*), except that with strain WIS 51–20 potassium or ammonium phenylacetate was added every 24 hr., beginning about 40 hr. after inoculation to give a final concentration of 0·1% instead of 0·2%.

Penicillin production and purity of the isolated penicillin *N*-ethylpiperidine salt were determined by cup-plate bioassay with *Bacillus subtilis* (I.C.I. strain, NCTC 8241) as test organism (Humphrey & Lightbown, 1952), and international standard sodium benzylpenicillin (1670 i.u./mg.) or its *N*-ethylpiperidine salt as the appropriate standard. Further details of individual fermentations are given in Table 1.

Washed mycelium experiments. Washed mycelium of *P. chrysogenum* WIS 51–20, prepared as described by Halliday & Arnstein (1956), was used at a concentration of 0·1 g. (wet wt.)/ml. of medium, the dry wt. being 10–15% of the wet wt. The mycelium and incubation medium, which contained 0·005% potassium phenylacetate and D- or L-[*carboxy*-^{14}C]-valine (50 or 200 μg./ml.), were incubated in conical flasks (250 ml. or 500 ml. for 25 ml. and 50 ml. of medium respectively) at 24° on a rotary shaker (250 rev./min.). Carbon dioxide was collected as $BaCO_3$ in some experiments by incubating the washed mycelium in a closed flask in an atmosphere of O_2 and passing CO_2-free air through the medium and through a trap containing saturated aqueous $Ba(OH)_2$ at the end of the incubation. At the end of the experiment, the mycelium was filtered off and washed with 0·1 M phosphate buffer (pH 6·9). The filtrate and washings were diluted to a known volume and assayed for penicillin activity. Carrier valine (approx. 40 mg./flask) and sodium benzylpenicillin (approx. 40 mg./flask) were added to the solution and penicillin was isolated in the usual way by extraction with ether at pH 2 and conversion into the *N*-ethylpiperidine salt, which was recrystallized to constant radioactivity. The aqueous solution remaining after ether

extraction was brought to pH 7 and evaporated to dryness *in vacuo*. The residue was dissolved in water and valine was isolated by adsorption on Zeo-Karb 215 ion-exchange resin (Permutit Co. Ltd., London) (approx. 5–10 g.) followed by elution with 0·2 M-NH_3 soln. The valine obtained by evaporation of the eleunt was recrystallized to constant radioactivity. The purity of the recrystallized product was checked by paper chromatography.

The mycelium from each flask was further washed with water, dried at room temperature over P_2O_5 and assayed directly for radioactivity.

Isolation and degradation methods

Penicillin. At the end of the fermentation, usually 120–140 hr. after inoculation, benzylpenicillin was isolated from the cooled, filtered broth by ether extraction at pH 2 and concentrated by successive partitioning between ether and buffer (pH 7) at 4° (Smith & Hockenhull, 1952). Finally, the *N*-ethylpiperidine salt was prepared and recrystallized from chloroform–acetone till the radioactivity remained unchanged.

Penicillin was degraded by heating with 0·1 N-H_2SO_4 at 100° essentially as described earlier (Arnstein & Grant, 1954*a*, *b*), except that conversion of the *N*-ethylpiperidine salt into the sodium salt was found to be unnecessary and was omitted. In addition, yields of *iso*propylidenepenicillamine were improved considerably by evaporating the solution of penicillamine hydrochloride, obtained from the mercury mercaptide derivative by treatment with H_2S, by freeze-drying rather than by vacuum distillation. With these two modifications it was possible to isolate pure *iso*propylidenepenicillamine (m.p. 191°) and penilloaldehyde 2:4-dinitrophenylhydrazone (m.p. 195–196°) from as little as 150 mg. of the *N*-ethylpiperidine salt of benzylpenicillin.

For ^{15}N analysis of the penilloaldehyde, dilution with extraneous nitrogen was minimized by preparing the *p*-bromophenylhydrazone derivative instead of the 2:4-dinitrophenylhydrazone. *p*-Bromophenylhydrazine hydrochloride (1%, w/v) in 2 N-HCl was added directly to the solution of penilloaldehyde obtained by degradation of penicillin with acid and $HgCl_2$, excess of the latter having been removed with H_2S. The product was filtered off, washed with water and dried (yield, 36%). After recrystallization from benzene, *penilloaldehyde* p-*bromophenylhydrazone*, m.p. 137°, was obtained. (Found: C, 55·4; H, 4·5; N, 12·1; Br, 22·7; $C_{16}H_{16}ON_3Br$ requires C, 55·5; H, 4·7; N, 12·1; Br, 23·1%.)

Penicillamine and valine. The direct oxidative decarboxylation of either penicillamine disulphide or penicillaminic acid with ninhydrin or chloramine T did not give good yields of the corresponding aldehydes, although the amount of CO_2 was satisfactory. Desulphurization of *iso*propylidenepenicillamine to valine, followed by treatment with ninhydrin, was, however, successful. *iso*Propylidenepenicillamine (50 mg.) in water (5 ml.) was boiled under reflux for 10 min. with 0·5 ml. of a suspension of Raney nickel in water (Mozingo, Wolf, Harris & Folkers, 1943). The reaction mixture was cooled, filtered and evaporated to dryness. In trial experiments, valine was found to be the only amino acid present, as shown by paper chromatography with two solvents [phenol–water, 5:2 (v/v) (with NH_3) and amyl alcohol–pyridine–water, 7:7:6 (by vol.)]. The above residue was therefore dissolved in 10 ml. of M potassium phosphate buffer (pH 5·8) and the solution was boiled briefly while CO_2-free N_2 was passed through. Ninhydrin (100 mg.) was then added and the solution boiled for a further 45 min. *iso*Butyraldehyde and CO_2 were collected in traps containing a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl and saturated $Ba(OH)_2$ respectively, and isolated as *iso*butyraldehyde 2:4-dinitrophenylhydrazone, m.p. 179–180° (89 mg., 73%) and $BaCO_3$ (17 mg., 40%).

Valine was oxidatively degraded by decarboxylation with either ninhydrin or chloramine T.

Amino acids from mycelial protein. The dried mycelium was defatted by boiling with ethanol and ether. The fat-free product was extracted with 5% (w/v) trichloroacetic acid (25 ml./g.) for 30 min. at room temp., 15 min. at 90° and again 30 min. at room temp. The residue was washed with ethanol and ether and dried at 100°. A portion of the dried protein-rich residue (5 g.) was hydrolysed with 6 N-HCl (50 ml.) at 100–105° for approx. 18 hr. in a sealed tube. The filtered hydrolysate was evaporated to dryness *in vacuo*, the residue dissolved in water (approx. 50 ml.), and the solution neutralized with Na_2CO_3. The amino acids were precipitated by adding alternately small portions of 10% Na_2CO_3 and 25% mercuric acetate (cf. Neuberg & Kerb, 1912). The free amino acids were regenerated by suspending the ppt. in dil. HCl and passing H_2S. The mixed amino acids were chromatographed on a column (60 cm. × 3 cm.) of Dowex 50 or Zeo-Karb 225, which was eluted with 1·5 N- and 2·5 N-HCl (cf. Stein & Moore, 1949). The appropriate fractions were evaporated to dryness and the separated amino acids were adsorbed on short columns of Zeo-Karb 215 resin (H form; approx. 1 g. of resin/10 mg. of N). The columns were washed with water until the effluent was neutral and the amino acids were eluted with 0·2 M-NH_3 soln. After evaporation of the solution to dryness, the amino acids were crystallized from aqueous ethanol and recrystallized to constant radioactivity. The purity of each amino acid was checked by paper chromatography.

Isotope estimations

Radioactivity measurements. Compounds were mounted on 1 cm.^{2} polythene disks and counted as samples of 'infinite thickness', a thin end-window Geiger–Müller tube (Popják, 1950) being used. All counts were compared with a poly[^{14}C]methyl methacrylate sheet reference standard obtained from the Radiochemical Centre, Amersham. The standard error in all determinations is less than 5%.

Determination of ^{15}N. Samples were assayed as in previous work (Arnstein & Grant, 1954*b*) with a 60° mass spectrometer. The accuracy of ^{15}N estimations is ±0·003 atom % excess.

Labelled compounds

Uniformly labelled L-[^{14}C]*valine*, DL-[α-^{14}C]*valine and* DL-[carboxy-^{14}C]*valine.* These compounds were obtained from the Radiochemical Centre, Amersham. Uniformly labelled valine will be abbreviated [U-^{14}C]valine in this paper.

Resolution of DL-[carboxy-^{14}C]*valine.* DL-[*carboxy*-^{14}C]-Valine (6·3 mg., 0·1 mc) was diluted with inactive DL-valine (3 g.), converted into the *N*-formyl derivative in 74% yield by formylation with formic acid–acetic anhydride (du Vigneaud & Patterson, 1935), and resolved as the brucine salt (Fischer, 1906). The molecular rotation of the L-[*carboxy*-^{14}C]valine was +61·6±2° (*c* = 0·604 in acetic acid), that of D-[*carboxy*-^{14}C]valine was −55·2±2° (*c* = 0·604 in acetic acid). Under the same conditions, inactive L- and D-valine

obtained from Roche Products Ltd. had identical molecular rotations, but the best literature value is somewhat higher (72·6°; Greenstein, 1954).

DL-[^{15}N]*Valine*. The synthesis of [^{15}N]valine was essentially similar to that used by Adams & Tolbert (1952) for preparing [α-^{14}C]valine. Hippuric acid was prepared in 94% yield from [^{15}N]glycine (4·1 g., 16 atom % excess ^{15}N) by the method of Steiger (1944) and converted into 2-phenyl-4-*iso*propylidene-5-ox[^{15}N]azolone (Cornforth, 1949) in 64% yield. Reduction of the oxazolone (4·64 g.) with P–HI (cf. Adams & Tolbert, 1952) yielded valine, which was purified by adsorption on Zeo-Karb 215 resin (H form, 1 g./10 mg. of N) and elution with 0·2 M-NH_3 soln. after the column had been well washed with water. The alkaline solution was evaporated to dryness *in vacuo* and DL-[^{15}N]valine was crystallized from aqueous ethanol (1·87 g., 66%; overall yield from glycine, 40%).

DL-β-*Hydroxy*[carboxy-^{14}C]*valine*. A solution of *iso*butylmagnesium bromide (0·25 mole) in ether (820 ml.) was allowed to react with $^{14}CO_2$ generated from $Ba^{14}CO_3$ (50 g.; 223·8 μc/mole), as in the method given by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). At the end of the reaction the mixture was poured on crushed ice, and a cooled solution of conc. H_2SO_4 (30 ml.) in water (250 ml.) was added with stirring. The aqueous layer was saturated with NaCl, the ether was separated and the aqueous solution was extracted twice with ether (200 ml.). The combined ether solutions were washed twice with saturated NaCl (100 ml.), dried (Na_2SO_4) and evaporated. The residue was distilled at atmospheric pressure, giving [*carboxy*-^{14}C]*iso*valeric acid (11·9 g.; 47·5%).

The *iso*valeric acid (10·8 g.) was brominated in the presence of dry red P (1·32 g.) by the slow addition of Br_2 (12 ml.) at room temp. Finally, the reaction mixture was heated on the steam bath for 24 hr. and then added dropwise to hot water (40 ml.). After heating on the steam bath for 1 hr., it was cooled and extracted three times with ether (2 × 25 ml.; 10 ml.). The ether extracts were combined, washed with saturated NaCl, dried (Na_2SO_4) and evaporated. The residue was distilled at 25 mm., giving 16·1 g. (89%) of crude α-bromo[*carboxy*-^{14}C]*iso*valeric acid.

The crude acid was refluxed with 3 N sodium methoxide in methanol (128 ml.) for 6 hr. (cf. Owen, 1949). Water (300 ml.) was added to the cold solution and methanol was removed by distillation *in vacuo*. The solution was acidified with conc. HCl and extracted with ether. After evaporation of the ether, the oily residue was crystallized by adding water. Yield of ββ-dimethyl[*carboxy*-^{14}C]acrylic acid, m.p. 69°, 4·1 g. (46·5%; specific radioactivity 203·2 μc/mole).

ββ-Dimethyl[*carboxy*-^{14}C]acrylic acid (4 g.) was converted into α-bromo-β-methoxy[*carboxy*-^{14}C]*iso*valeric acid as described by Rufenach (1952). Yield after recrystallization from light petroleum (b.p. 60–80°) 5·2 g. (62%); m.p. 75–78°; specific radioactivity 225·8 μc/mole.

The product was aminated by heating at 100° for 2 hr. with conc. NH_3 soln. (56 ml., sp.gr. 0·88) in a sealed tube. The solution was evaporated to dryness and the residue was redissolved in hot water (50 ml.). After filtration, ethanol (200 ml.) was added, when α-amino-β-methoxy[*carboxy*-^{14}C]*iso*valeric acid (β-methoxyvaline) crystallized. The product (2 g., 56%, m.p. about 260°) contained no halogen and was found to be chromatographically pure [Whatman no. 3 MM paper with phenol–water, 5:2 (v/v) (with NH_3) as solvent].

Hydrolysis of α-amino-β-methoxy*iso*valeric acid with HBr (Abderhalden & Heyns, 1934) for 1 hr. or more was accompanied by appreciable destruction of the product, β-hydroxyvaline, but 0·5 hr. was found to be sufficient for complete hydrolysis. β-Methoxy[*carboxy*-^{14}C]valine (2 g.) was therefore refluxed for 0·5 hr. with 48% HBr (20 ml.), water was added and HBr removed by evaporation *in vacuo*. The dry residue was dissolved in water and the solution passed through a column of Zeo-Karb 215 resin (50 g., H form). The column was washed with water till the pH of the effluent was neutral and then eluted with 0·2 M-NH_3 soln. The eluate was evaporated to dryness and the residue was

Table 1. *Experimental details of fermentations*

Solutions of the labelled amino acids in about 6 ml. of water were sterilized by Seitz filtration and added in approximately equal portions at the stated times after inoculation of the medium. The amount of penicillin is that present at the end of the fermentation, which was usually the maximum synthesized. *Penicillium chrysogenum* WIS 48–701 was used in Expts. 2, 3, 5 and 6, strain WIS 51–20 in the other experiments. The volume of the fermentation was either 1 l. (Expts. 1–3 and 9) or 1·5 l. (Expts. 4–8 and 10).

	Labelled precursor added						
			Isotope content				
Expt.	Amino acid	Total wt. (mg.)	(μc of ^{14}C/mole)	(atom % excess ^{15}N)	Times of addition of precursor (hr.)	Duration of fermentation (hr.)	Yield of penicillin (i.u./ml.)
1	DL-[α-^{14}C:^{15}N]Valine	224	223	4·63	0, 22, 89, 118, 142	167	248
2		281	510	4·34	0, 25, 46, 65, 94, 113	120	237
3		175	413	4·43	40, 63, 87	153	112
4		348	355	4·50	41, 65, 89	115	428
5		439	339	4·65	45, 67, 91	114	120
6		285	99·6	4·70	46, 96	120	105
7		330	127	4·70	72, 100, 118	160	206
8	L-[U-^{14}C]Valine + DL-[^{15}N]valine*	439	1830	15·52	41, 65, 88, 112	117	421
9	DL-β-Hydroxy[*carboxy*-^{14}C]valine	285	197	—	42, 65, 88, 112	112	147
10		497	197	—	46, 70, 93, 116	134	323

* The mixture contained 0·4 mg. of L-[U-^{14}C]valine (28·5 μc/mg.) and 439 mg. of DL-[^{15}N]valine.

crystallized from aqueous ethanol, giving 1·19 g. (65%) of β-hydroxy[*carboxy*-^{14}C]valine, m.p. 220°, with decomposition. The specific radioactivity was 197·2 μc/mole and the overall yield from $^{14}CO_2$ was 4%. The purity of the product was checked by paper chromatography [Whatman no. 3 MM paper; phenol–water, 5:2 (v/v) (with NH_3)], and by constant radioactivity after recrystallization from aqueous ethanol.

RESULTS

Utilization of valine for biosynthesis of penicillin and mycelial protein

The intact utilization of the carbon chain of valine for the biosynthesis of the penicillamine moiety of penicillin [see formula (I)] is indicated by the distribution of ^{14}C in the mycelial valine and in the penicillin isolated from a fermentation to which L-[U-^{14}C]valine had been added (Table 2). As in the experiments with DL-[α-^{14}C]valine (cf. Table 6), nearly all the radioactivity (95%) in the penicillin was located in the penicillamine portion of the molecule. Further degradation of penicillamine showed that the average radioactivity per carbon atom (6·5 μc) was almost identical with the average radioactivity of $C_{(2)}$, $C_{(3)}$, $C_{(4)}$ and $C_{(4')}$ (6·65 μc) and similar to that of the carboxyl-carbon atom (5·2 μc). The latter value appears to be somewhat low, owing possibly to accidental dilution of the CO_2 from the carboxyl-carbon atom by a little extraneous CO_2. A similar partial degradation of the mycelial valine and of the valine added to the fermentation shows that the carboxyl-carbon atom of valine contains in each case about 20% of the total radioactivity.

Table 2. *Comparison of* ^{14}C *distribution in valine and penicillamine*

Uniformly ^{14}C-labelled L-valine was added to a fermentation of *P. chrysogenum*, WIS 51–20 (Expt. 8, Table 1). Figures in parentheses give the distribution of ^{14}C in the degradation products as a percentage of the initial radioactivity before degradation.

Compound degraded	Radioactivity (μc/mole)	Radioactivity in degradation products (μc/mole): *iso*Butyraldehyde 2:4-dinitrophenylhydrazone ($C_{(2)}$–$C_{(4')}$)	Radioactivity in degradation products (μc/mole): $BaCO_3$ ($C_{(1)}$)
Valine added	1830	1500 (81)	385 (21)
Valine isolated from mycelial protein	63·2*	52·7 (83)	13·4 (21)
Penicillamine obtained by degradation of penicillin	32·5*	26·6 (82)	5·2 (16)

* After dilution with carrier.

Table 3. *Conversion of labelled valine and β-hydroxyvaline into penicillin and mycelial protein valine*

Penicillin was isolated as the *N*-ethylpiperidine salt, which was analysed for ^{15}N. The reported ^{15}N content has therefore been corrected for dilution by one atom of non-isotopic nitrogen by multiplying the observed values by 1·5. For experimental details and isotope content of the labelled amino acids added to the fermentations see Table 1 and text. – Signifies value not determined.

Expt.	Labelled amino acid added	Compound isolated	Isotope content of isolated compound: ^{14}C (μc/mole)	Isotope content of isolated compound: ^{15}N (atom % excess)	Dilution of added isotope: ^{14}C	Dilution of added isotope: ^{15}N
1	DL-[α-^{14}C:^{15}N]Valine	Penicillin	28·9	0·120	7·7	39
		Mycelial valine	23·7	0·119	9·4	39
2		Penicillin	75·8	–	6·7	–
3		Penicillin	84·1	–	4·9	–
4		Penicillin	37·2	0·081	9·6	56
		Mycelial valine	25·4	0·077	14	58
5		Penicillin	37·1	–	9·1	–
6		Penicillin	9·5	–	10·5	–
8	L-[U-^{14}C]Valine + DL-[^{15}N]valine*	Penicillin	260	0·404†	7·0	38·5
		Mycelial valine	199·5	0·464	9·2	33·5
10	DL-β-Hydroxy[*carboxy*-^{14}C]valine	Penicillin	5·71‡	–	35	–
		Mycelial valine	1·14	–	173	–

* See footnote to Table 1.

† Calculated from the ^{15}N analysis of penicillin *N*-ethylpiperidine salt which had been diluted with a known amount of carrier.

‡ In a second experiment with DL-β-hydroxy[*carboxy*-^{14}C]valine (Expt. 9, Table 1) the radioactivity of the isolated penicillin was also very low (about 2 μc/mole) but was not measured accurately.

In these experiments, mixtures of DL-[^{15}N]valine and either DL-[α-^{14}C]valine or L-[U-^{14}C]valine were used as precursors (see Table 1), and the ^{15}N and ^{14}C contents of penicillin and valine from the mycelial protein are given in Table 3. The dilutions of ^{14}C for the biosynthesis of penicillin and mycelial protein were approximately 8 (range 4·9–10·5) and 11 (range 9·2–14) respectively. The rather wide range is probably due to differences in the times of addition of labelled valine to the fermentation and to variation in penicillin production (see Table 1). In all experiments, however, isotopic nitrogen was incorporated into penicillin and mycelial valine only with much greater dilution than was isotopic carbon, the average dilution being 45 and 50 respectively.

Degradation of the penicillin showed that both the penicillamine nitrogen atom and the side-chain nitrogen atom contained ^{15}N (Table 4). In three cases (I–III) the ^{15}N content of the penicillamine was not significantly above that of the average for both nitrogen atoms of penicillin, but in one case (IV) it was twice that of the side-chain nitrogen atom. It is noteworthy that in this experiment the isotope content of the mycelial valine was exceptionally high (see Table 3, Expt. 8), which may

Table 4. *Distribution of isotopic nitrogen in penicillin derived from* DL-[^{15}N]*valine*

For the degradation of penicillin, samples of penicillin *N*-ethylpiperidine salt from the following experiments were combined with carrier when necessary: I—Expts. 2, 3, 5 and 6; II—Expts. 1 and 7; III—Expt. 4; IV—Expt. 8. The ^{15}N content of the penicillin is the *average* value for both nitrogen atoms. All ^{15}N analyses have been corrected for non-isotopic nitrogen present in the derivatives analysed. – Signifies value not determined.

Substance	^{15}N Content (atom % excess) I	II	III	IV
Penicillin (average of both N atoms calc. from analysis of *N*-ethylpiperidine salt)	0·054±0·005	0·047±0·005	0·081±0·005	0·155±0·005
Penicillamine (isolated as *iso*propylidene derivative)	{0·044±0·003 0·047±0·003}	{0·046±0·003 0·048±0·003}	0·092±0·003	0·203±0·003
Penilloaldehyde (calc. from analysis of 2:4-dinitrophenylhydrazone)	–	–	–	0·105±0·015
Penilloaldehyde (calc. from analysis of *p*-bromophenylhydrazone)	–	–	–	0·078±0·009 0·102±0·009

Table 5. *Comparison of* ^{15}N *content of valine and some other amino acids isolated from mycelial protein*

The results have been calculated on the basis of 100 atom % excess ^{15}N in the added valine in order to compare the different experiments. – Signifies value not determined.

Amino acid isolated	^{15}N Content (atom % excess) Expt. 1	Expt. 4	Expt. 8
Alanine	1·66	1·45	1·99
Aspartic acid	1·53	–	–
Glutamic acid	1·66	1·29	–
Glycine	1·47	1·45	1·67
Valine	2·53	1·72	2·99

Table 6. *Relative incorporation of* ^{14}C *and* ^{15}N *from labelled valine into penicillin and mycelial protein*

The experimental results have been taken from Tables 1, 2, 4 and 7. In Expt. 4 DL-[α-^{14}C:^{15}N]valine was added to a fermentation, in Expt. 8 a mixture of L-[U-^{14}C]- and DL-[^{15}N]valine was used. The ^{14}C content is expressed as μc/mole, the ^{15}N content as atom % excess. The figures in parentheses in columns 6 and 7 give the change in the ^{14}C/^{15}N isotope ratio, obtained by dividing the ^{14}C/^{15}N ratio of the isolated valine or penicillamine by that of the valine added to the fermentation. Unless otherwise indicated, ^{15}N analyses are accurate to within ±0·003 and the standard error of the ^{14}C assays is about ±5%. These limits have been used to estimate the accuracy of the isotope ratios given in columns 6 and 7.

Compound	Isotope content Expt. 4 ^{14}C	^{15}N	Expt. 8 ^{14}C	^{15}N	^{14}C/^{15}N isotope ratio Expt. 4	Expt. 8
Added valine	355	4·50	1830	15·522	79±4	118±6
Mycelial valine	25	0·077	199·5	0·464	330±31 (4·2±0·6)	430±24 (3·65±0·4)
Penicillamine	38·2	0·092	246*	0·528*	415±37 (5·3±0·7)	465±28 (3·9±0·4)

* Calc. from isotope content of material diluted with carrier; ^{15}N analysis accurate to ±0·0075.

explain the relatively high incorporation of ^{15}N into the penicillamine nitrogen atom.

These results indicate that valine is reversibly deaminated before its incorporation into penicillin and mycelial protein. The deamination of valine is also shown by the extensive incorporation of isotopic nitrogen from [^{15}N]valine into other amino acids (Table 5). Thus alanine, glycine, glutamic acid and aspartic acid isolated from the mycelial protein of fermentations to which DL-[^{15}N]valine had been added contained relatively large amounts of ^{15}N. In one experiment (Expt. 4) the ^{15}N content of mycelial glycine and alanine was only 16 % less than that of valine.

A comparison of DL- and L-valine as penicillin precursors is given in Table 6. The incorporation of ^{14}C and ^{15}N into the penicillamine moiety of penicillin closely parallels that into mycelial valine in both experiments. Since in one experiment (Expt. 8) only the L-form of the added valine was labelled with ^{14}C, any preferential utilization of D-valine would have resulted in a marked decrease in the ^{14}C:^{15}N isotope ratio of the penicillamine moiety compared with that of mycelial valine. In each experiment, the ^{14}C:^{15}N ratio of mycelial valine is, however, identical with that of penicillamine within the limits of accuracy of the isotope analyses, indicating that D-valine is not utilized preferentially.

Penicillin biosynthesis from ^{14}C-labelled L- and D-valine by washed mycelium

The radioactivity of penicillin synthesized by washed mycelium in the presence of L- or D-[*carboxy*-^{14}C]valine is shown in Table 7. When the labelled valine was added at a final concentration of 0·005 %, L-[*carboxy*-^{14}C]valine gave rise to penicillin of higher radioactivity than that obtained from D-[*carboxy*-^{14}C]valine under identical conditions, although there was no significant difference in the rate of penicillin biosynthesis. The greatest difference in the conversion of L- and D-valine into penicillin was observed with short (1 or 2 hr.) incubation periods. Increase of the concentration of labelled valine to 0·02 % (Expt. WM 3) resulted in almost equal utilization of L- and D-valine in 4 hr.

Table 7 also gives the radioactivity in the CO_2 produced during incubation of labelled valine and that left in the mycelium after 1, 2 and 4 hr. The conversion of L-valine into CO_2 is much greater than that of the D-isomer. Metabolism of L-valine to CO_2 is, however, complete within 2 hr., since the radioactivities of CO_2 after 2 and 4 hr. are similar. On the other hand, metabolism of D-valine to CO_2 is very slow at first but increases between 2 and 4 hr.

In both experiments with 0·005 % L-[*carboxy*-^{14}C]-valine more radioactivity is present in the mycelium after 1 hr. than after 2 or 4 hr. With D-[*carboxy*-^{14}C]-

Table 7. *Incorporation of* L- *and* D-[carboxy-^{14}C]*valine into penicillin, mycelium and carbon dioxide by washed mycelium of* P. chrysogenum

All results are calculated for 100 ml. of medium (10 g. of wet mycelium). The specific radioactivity of the L- and D-[*carboxy*-^{14}C]valine was 3·32 μc/m-mole. Freshly harvested mycelium of *P. chrysogenum* WIS 51–20 was used for experiments WM 1 and WM 3. The mycelium used in experiment WM 2 had been stored for 1 day under O_2 at 0°.

Expt.	Labelled valine added (%)	Time of incubation (hr.)	Radioactivity in Mycelium (μmc)	Radioactivity in CO_2 (μmc)	Yield of penicillin (i.u./ml.)	Specific radioactivity of penicillin (μc/m-mole)
			L-[carboxy-^{14}C]*valine added*			
WM 1	0·005	1	42	15	4·2	1·52
		2	25	24	7·2	1·50
		4	31	26	10·1	1·16
WM 2	0·005	1	38	14	3·6	1·56
		2	19	30	6·3	1·14
		4	24	26	10·6	0·92
WM 3	0·02	4	157	98	7·2	1·27
			D-[carboxy-^{14}C]*valine added*			
WM 1	0·005	1	8	1·1	3·7	0·438
		2	11	4·8	6·9	0·634
		4	20	12·0	11·3	0·708
WM 2	0·005	1	8	1·1	3·4	0·398*
		2	12	2·0	6·2	0·439
		4	22	11·0	10·4	0·632
WM 3	0·02	4	175	52·0	7·6	1·25

* Sample not recrystallized.

valine the radioactivity of the mycelium increases throughout the incubation period. The difference in radioactivity incorporated into the mycelium from L- or D-[*carboxy*-^{14}C]valine was greatest after incubation for 1 hr. and decreased with time and thus paralleled that incorporated into penicillin.

With both L- and D-valine, a fourfold increase in concentration resulted in an approximately similar increase in their conversion into CO_2 at 4 hr., but the amount of radioactivity left in the mycelium was increased more than fourfold. No adequate explanation can at present be given for this finding.

Uptake of L- *and* D-*valine by mycelial cells*

The uptake by washed mycelium of L-valine, added to the incubation medium at a concentration of either 0·005 or 0·02%, was essentially complete after 1 hr., but D-valine was absorbed only slowly, 40% or more being left in the extracellular medium after 4 hr. (Fig. 1). The initial rate of uptake of L-valine appears to be at least 0·1 mg./min./g. of wet mycelium, whereas that of D-valine is only 0·01 mg./min./g. of mycelium.

In Fig. 2, the results given in Table 7 for the conversion of D-valine into penicillin have been calculated as a percentage of the conversion of L-valine into penicillin and compared with the uptake of D-valine, also expressed as a percentage of that of L-valine. It is clear that the less efficient utilization of D- compared with L-valine for penicillin biosynthesis is quantitatively accounted for by the slower uptake of the D-stereoisomer.

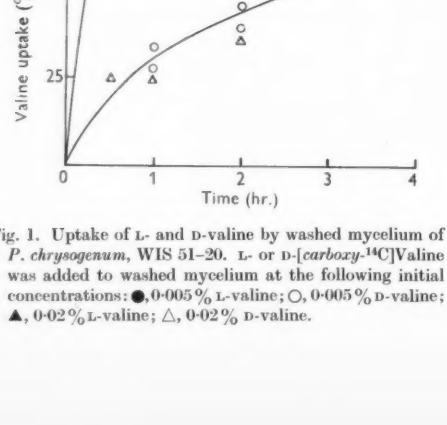


Fig. 1. Uptake of L- and D-valine by washed mycelium of *P. chrysogenum*, WIS 51–20. L- or D-[*carboxy*-^{14}C]Valine was added to washed mycelium at the following initial concentrations: ●, 0·005% L-valine; ○, 0·005% D-valine; ▲, 0·02% L-valine; △, 0·02% D-valine.

Comparison of valine and β-hydroxyvaline as penicillin precursors

Labelled β-hydroxyvaline was incorporated into penicillin only with a much greater dilution of isotope than valine (Table 3). Degradation of the penicillin derived from DL-β-hydroxy[*carboxy*-^{14}C]-valine shows that most of the isotope is located in the β-lactam carboxyl-carbon atom ($C_{(7)}$). The penicillamine moiety contained only 16% of the total radioactivity, whereas ^{14}C-labelled valine is converted into penicillin containing 94–100% of the total radioactivity in this portion of the molecule (Table 8). It is clear, therefore, that β-hydroxyvaline is not an intermediate in the utilization of valine for penicillin biosynthesis.

A comparison of the radioactivity of penicillin with that of several amino acids isolated from the mycelial protein of fermentations with labelled valine and β-hydroxyvaline (Table 9) also shows that hydroxyvaline is metabolized quite differently from valine. In the three experiments with labelled valine, the radioactivity of the mycelial valine was about 70% of that of the penicillin and about 30 times that of any of the other mycelial amino

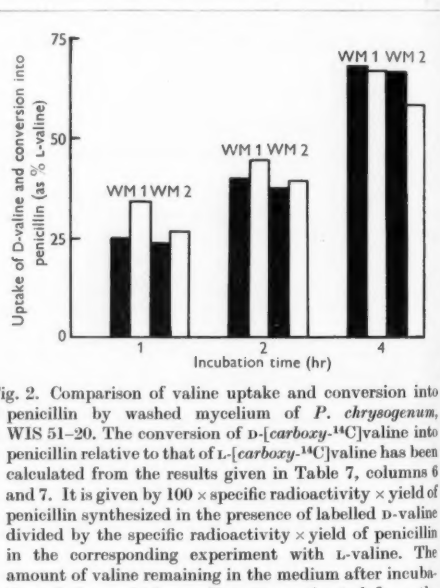


Fig. 2. Comparison of valine uptake and conversion into penicillin by washed mycelium of *P. chrysogenum*, WIS 51–20. The conversion of D-[*carboxy*-^{14}C]valine into penicillin relative to that of L-[*carboxy*-^{14}C]valine has been calculated from the results given in Table 7, columns 6 and 7. It is given by 100 × specific radioactivity × yield of penicillin synthesized in the presence of labelled D-valine divided by the specific radioactivity × yield of penicillin in the corresponding experiment with L-valine. The amount of valine remaining in the medium after incubation of the washed mycelium was estimated from the radioactivity of the valine isolated after addition of carrier (see Experimental section). Valine uptake was obtained by subtracting this figure from the amount of valine added initially. ■, Penicillin biosynthesis from D-valine as % from L-valine; □, uptake of D-valine as % of L-valine.

Table 8. *Distribution of* ^{14}C *in penicillin from fermentations containing* ^{14}C*-labelled valine or hydroxyvaline*

Penicillin was counted as the *N*-ethylpiperidine salt, penicillamine as the *iso*propylidene derivative, penilloaldehyde either as the 2:4-dinitrophenylhydrazone or the *p*-bromophenylhydrazone and the carboxyl carbon of the β-lactam ring ($C_{(7)}$) as $BaCO_3$. For other experimental details see text. When necessary the penicillin was diluted with carrier before degradation. The degradation of penicillin from Expt. 8 was carried out in duplicate. – Signifies value not determined.

Expt.	Radioactive amino acid tested as precursor	Radioactivity (μc/mole) in penicillin and degradation products: Penicillin (*A*)	Penicillamine (*B*)	Penilloaldehyde	$C_{(7)}$	Radioactivity in penicillamine (%) (100*B*/*A*)
2, 3, 5, 6	DL-[α-^{14}C]Valine	16·1	16·1	–	Inactive	100
1, 7		6·2	6·0	Inactive	Inactive	97·5
4		37·2	38·2	–	Inactive	103
8	L-[U-^{14}C]Valine	94·6*	90·9	0·6	4·3	95·5
			89·0	1·2	–	94·0
10	DL-β-Hydroxy[*carboxy*-^{14}C]valine	1·9*	0·3	Inactive	1·6	15·8

* After dilution with carrier.

Table 9. *Conversion of valine and β-hydroxyvaline into penicillin and amino acids by fermentations of* P. chrysogenum

In order to compare different experiments the radioactivity of the isolated compounds has been calculated for a specific radioactivity of the added amino acid (1 mc/mole). The amino acids were isolated from the mycelial protein. – Signifies value not determined.

Compound isolated	Radioactivity (μc/mole): Valine expt. (1)	Valine expt. (4)	Valine expt. (8)	Hydroxyvaline expt. (10)
Alanine	0·40	0·20	3·32	1·1
Aspartic acid	0·54	–	–	0·48
Glutamic acid	1·08	1·27	–	Inactive
Glycine	0·29	0·24	1·02	30·4
Valine	106	71·5	109	5·7
Penicillin	130	105	142	29

acids isolated. With labelled hydroxyvaline, however, the highest radioactivity was found in the mycelial glycine. Penicillin, labelled largely in the β-lactam portion as already mentioned, had a radioactivity similar to that of the mycelial glycine. The radioactivity of the mycelial valine was only about 20% of that of the glycine and thus corresponded approximately to that of the penicillamine moiety of penicillin (Table 8).

DISCUSSION

The present experiments with [α-^{14}C]valine and uniformly ^{14}C-labelled valine confirm our earlier results with DL-[γγ′-^{14}C]valine (Arnstein & Grant, 1954*a*), which indicated that valine is used by *Penicillium chrysogenum* as a precursor of the penicillamine moiety of penicillin (I). Utilization of the intact carbon chain of valine has now been demonstrated by the equal incorporation of uniformly labelled valine into the carboxyl carbon atom and $C_{(2)}$–$C_{(4')}$ of penicillamine. This conclusion is in agreement with the work of Stevens, Vohra & De Long (1954), who showed that DL-[*carboxy*-^{14}C]-valine gives rise to penicillin labelled in the carboxyl-carbon atom of penicillamine.

The low ^{15}N content of both penicillin and mycelial valine isolated from fermentations to which DL-[^{15}N]valine had been added indicates that valine is oxidatively deaminated or transaminated, presumably to dimethylpyruvic acid (α-oxo*iso*-valeric acid), at a rate which must be considerably faster than penicillin or protein synthesis. *Penicillium chrysogenum* contains both an L- and a D-amino acid oxidase (Knight, 1948; Emerson, Puziss & Knight, 1950), and dimethylpyruvic acid has been isolated from a related organism, *Aspergillus niger* (Ramachandran & Walker, 1951). It is noteworthy, however, that *A. niger* is apparently unable to synthesize dimethylpyruvate from valine.

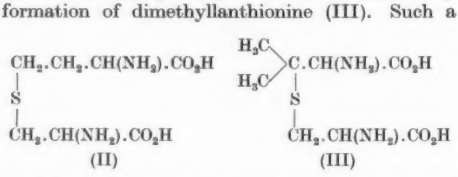
The relatively efficient incorporation of carbon-labelled valine into the mycelial protein implies the re-utilization of the carbon chain by amination of the keto acid to L-valine. The equally efficient utilization of carbon-labelled valine for penicillin biosynthesis does not, however, require the participation of D-valine as an obligatory intermediate. Although the relevant carbon atom of penicillin [$C_{(3)}$, formula (I)] has the D-configuration, it is conceivable that L-valine reacts with L-cysteine to give an intermediate which might subsequently be converted into penicillin by reactions involving the introduction of the D-configuration. Thus the following would be a possible biosynthetic pathway:

L-valine → L-cysteinyl-L-valine →
L-cysteinyl-D-valine → penicillin.

On the other hand, it should be pointed out that D-valine has not been excluded as a possible intermediate, since the preferential utilization of L-valine for penicillin synthesis by washed mycelium

is due to its faster uptake by the cells compared with that of D-valine. When allowance is made for the differences in uptake, both L- and D-valine appear to be converted into penicillin to an equal extent. Recently it has been reported that growing cultures of *P. chrysogenum* utilize D-valine for penicillin biosynthesis only after an initial lag period, whereas L-valine is used immediately (Stevens, Inamine & De Long, 1956). It was suggested that D-valine is therefore not a normal intermediate in penicillin synthesis, but possible differences in the uptake of the D- and L-amino acid by the cells were apparently not considered. In view of our present results it seems likely that this observed lag period in the utilization of D-valine must also be ascribed to its slower uptake.

In an attempt to elucidate the mechanism of penicillin biosynthesis from valine in more detail, β-hydroxyvaline was tested as a possible intermediate. By analogy with the formation of cystathionine (II) from cysteine by *Neurospora* (Horowitz, 1947), the thio ether link of penicillin might have been expected to be synthesized by condensation of cysteine with hydroxyvaline, as has been suggested by Hockenhull, Ramachandran & Walker (1949), possibly with the intermediary formation of dimethyllanthionine (III). Such a



(II) (III)

mechanism would require the incorporation of labelled hydroxyvaline into the penicillamine moiety with less dilution of isotope than that found with valine. Our experiments show, however, that DL-β-hydroxy[*carboxy*-^{14}C]valine is utilized much less efficiently than valine for penicillin biosynthesis. Moreover, the isotope was incorporated mainly into $C_{(7)}$ of penicillin (I). These results and the high ^{14}C content of glycine isolated from the mycelial protein suggest that hydroxyvaline is converted into penicillin only after breakdown to glycine and, presumably, acetone. Such a cleavage of the $\alpha\beta$ carbon–carbon bond of hydroxyvaline has been demonstrated in animal tissue (Vilenkina, 1949). Glycine is known to be an indirect precursor of the β-lactam ring of penicillin (Arnstein & Grant, 1954*a*), probably by the metabolic pathway glycine → serine → cysteine, and this mechanism would account for the labelling of $C_{(7)}$ of penicillin in the hydroxyvaline experiment.

Although β-hydroxyvaline itself has thus been excluded as a penicillin precursor a peptide such as cysteinyl-β-hydroxyvaline could still be an intermediate in penicillin biosynthesis, if it were synthesized by oxidation of cysteinylvaline rather than by reaction of cysteine and hydroxyvaline. Although this possibility may seem unlikely, several more obvious mechanisms have now been excluded. Thus $\beta\beta$-dimethyllanthionine is apparently not a penicillin precursor (Stevens, Vohra, Moore & De Long, 1954) and phenylacetylcysteine, which could give rise to phenylacetyldimethyllanthionine and thence penicillin without the intermediary formation of free dimethyllanthionine, is not utilized as such for penicillin biosynthesis (Arnstein, Clubb & Grant, 1954). The initial step of penicillin biosynthesis therefore probably involves the condensation of cysteine and valine by a peptide link, whereas the thio ether may be introduced subsequently by the intramolecular cyclization of an intermediate such as cysteinyl-β-hydroxyvaline or cysteinyl-$\alpha\beta$-dehydrovaline.

SUMMARY

1. Uniformly ^{14}C-labelled L-valine, DL-[α-^{14}C]-valine, DL-[^{15}N]valine and DL-β-hydroxy[*carboxy*-^{14}C]valine have been compared as penicillin precursors in fermentations of two strains of *Penicillium chrysogenum*. The utilization of valine for penicillin biosynthesis has also been studied with suspensions of washed mycelium to which L- or D-[*carboxy*-^{14}C]-valine was added.

2. Degradation of penicillin derived from uniformly ^{14}C-labelled valine shows that the carbon chain of the amino acid is used intact for biosynthesis of the penicillamine moiety of penicillin.

3. Valine labelled in the carbon chain with ^{14}C was incorporated into penicillin with much less dilution of isotope than was ^{15}N-labelled valine, indicating the rapid deamination of valine by a reversible reaction such as either an oxidative deamination or a transamination to dimethylpyruvic acid.

4. The conversion of L-valine into penicillin by washed mycelium is more efficient than that of D-valine. This difference appears to be due to the slower uptake of D-valine by the cells rather than to the preferential utilization of the L-enantiomorph for penicillin biosynthesis.

5. Experiments with β-hydroxy[*carboxy*-^{14}C]-valine show that this amino acid is not an intermediate in the biosynthesis of penicillin from valine. The distribution of isotope in penicillin and in certain amino acids of the mycelial protein suggests that hydroxyvaline is metabolized to glycine and, presumably, acetone.

6. The synthesis of DL-[^{15}N]valine and DL-β-hydroxy[*carboxy*-^{14}C]valine is described.

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Glycerylphosphorylcholine and Phosphorylcholine in Semen, and their Relation to Choline

By R. M. C. DAWSON
Biochemistry Department, A.R.C. Institute of Animal Physiology, Babraham, Cambridge

and T. MANN and I. G. WHITE
A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute and Department of Veterinary Clinical Studies, University of Cambridge

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Mammalian seminal plasma represents one of the richest natural sources of free choline and water-soluble choline compounds (Mann, 1954). However, as shown by Kahane & Lévy (1936, 1937), free choline accumulates in human seminal plasma only after ejaculation, as the result of a hydrolytic process which takes place in voided semen, on standing. Investigations on the nature of the choline precursor, which were carried out independently in the laboratories of Kahane in France (Kahane & Lévy, 1938, 1945, 1949; Diament, Kahane & Lévy, 1952, 1953; Diament, 1954, 1955) and of Lundquist in Denmark (Lundquist, 1946, 1947*a*, *b*, 1953), led to somewhat conflicting results, particularly as regards (i) the ratio between free choline and the simultaneously liberated inorganic phosphate, and (ii) the nature of the phosphorylated choline precursor. It is the purpose of this paper to report the results of an investigation of the nature and distribution of choline compounds in the semen and male accessory secretions of various species. As well as human semen, the study included material from monkey, ram, goat, bull, boar, stallion, rat, rabbit, hedgehog and cock.

EXPERIMENTAL

Collection of semen and of secretions from male accessory organs. Human semen from donors at the Fertility Clinic was kindly made available by Dr H. A. Davidson. The semen of ram, bull, boar, goat, rabbit and stallion was

collected by means of an artificial vagina (Walton, 1945); the two parts of semen, the sperm and seminal plasma, were separated by centrifuging. Cock semen was obtained by the method of abdominal massage (Burrows & Quinn, 1939). The secretory fluids from male accessory organs were obtained at autopsy.

Whenever possible semen was frozen in solid CO_2 (− 78°) immediately after ejaculation and stored at this temperature until it was used for determinations of glycerylphosphorylcholine (GPC) or phosphorylcholine. Evidence was obtained that in human semen there was a breakdown of phosphorylcholine during storage for a few days in a deep-freeze (− 15°).

Preparation of suspensions of washed spermatozoa. Ram semen (1·0–1·5 ml.) was diluted with 6–8 vol. of a diluent, in a conical centrifuge tube, the spermatozoa were separated by centrifuging at about 1000 *g*, and washed twice with 6–8 vol. of fresh diluent. The diluents were: (i) a calcium-free Ringer's solution, composed of 100 ml. of 0·9% NaCl, 4 ml. of 1·15% KCl, 1 ml. of 2·11% KH_2PO_4, 1 ml. of 3·82% $MgSO_4,7H_2O$ and 2 ml. of 1·3% $NaHCO_3$; (ii) the same solution but with phosphate omitted ('P-free Ringer solution'); (iii) a calcium-free Ringer's solution supplemented with 20 ml. of 0·25M phosphate buffer, pH 7·4 ('Ringer-phosphate', Mann, 1946); (iv) a 'phosphate-diluent' (White, Blackshaw & Emmens, 1954), containing 0·032M-NaH_2PO_4,H_2O, 0·048M-$Na_2HPO_4,12H_2O$, 0·040M-NaCl and 0·004M-KCl. The final concentration of spermatozoa in the washed sperm suspensions was about 3×10^8 cells/ml.

Chromatographic detection of GPC and phosphorylcholine. The sample of semen or seminal plasma (0·1–0·75 ml. according to the acid-soluble P content) was introduced into 4 ml. of boiling water, and maintained at 100° for 3 min. Ethanol (7 ml.) was added and the mixture centrifuged. The slightly turbid supernatant was shaken with 30 ml. of $CHCl_3$. The milky upper layer was centrifuged for 20 min. at 10 000 *g* and the clear supernatant was rapidly passed through a column of Amberlite IRC 50 (H) (8 cm. × 0·8 cm.²) to remove cations. The effluent (25 ml.) was shaken with 75 ml. of *iso*butanol and the clear aqueous layer which separated was adjusted to pH 7 with NH_3 soln., taken down to a small volume *in vacuo*, and then applied to the origin of the paper chromatogram. Sometimes, especially with bull, stallion and boar semen, it was difficult to apply the final solution to the chromatogram owing to the presence of a gummy material, but this had no noticeable effect on the quality of the chromatograms developed. The methods of chromatography used, and the detection of the P-containing spots, have been previously described (Dawson, 1954, 1955). The solvents used were phenol saturated with 0·1% NH_3 soln. in one dimension and ethanol–NH_3 soln. (sp.gr. 0·880)–water (6:3:1, by vol.) in the second. It was found that the best separations were obtained when the second solvent was run at a temperature below 20°. The GPC spot had approximate R_P values of 0·88 and 0·55 in the respective solvents, and those for phosphorylcholine were 0·89 and 0·27. The identity of the spots was confirmed by running the sample simultaneously with synthetic compounds as markers.

Determination of GPC and phosphorylcholine. The frozen sample was either rapidly thawed and then pipetted or it was weighed in the frozen state on a torsion balance. It was then introduced into 2 ml. of 0·5% serum albumin solution at 0° and immediately precipitated with 1 ml. of 10% (w/v) perchloric acid solution. Serum albumin was added to facilitate the precipitation of lipid material. The suspension was centrifuged and the supernatant filtered and stored at 0°. Two samples (1 ml.) were each mixed with 0·25 ml. of 5N-HCl; one was then kept at 0° and the other hydrolysed at 100° for 20 min. to release the acid-labile choline from GPC. The free choline in the unhydrolysed and hydrolysed samples was estimated by the method of Appleton, La Du, Levy, Steel & Brodie (1953), the choline being directly precipitated with the tri-iodide reagent. The entire estimation, including colorimetry, was carried out at 0°. Recovery of choline and GPC, with standards of these two substances, was linear between 0 and 200 μg. and 0 and 450 μg. respectively. Recovery of GPC added to dialysed ram seminal plasma was linear between 0 and 360 μg.

Phosphorylcholine was estimated in human semen samples by estimating the choline present before and after incubation for 90 min. at 37°. Under these conditions phosphorylcholine is completely hydrolysed by the acid phosphatase in the semen and GPC is not affected.

Isolation of GPC from ram seminal plasma. Seminal plasma (40 ml.), obtained from ram semen by centrifuging, was precipitated with 6 vol. of ethanol, and the filtered water-clear ethanolic extract concentrated *in vacuo* to a volume of 6 ml. After removing the small water-insoluble residue by centrifuging, the concentrate was poured, under constant stirring, into 6 vol. of acetone, and 1 hr. later the oily, brownish coloured bottom layer which formed on standing was separated from the colourless supernatant solution by decantation. The oily fraction was extracted with three successive 5 ml. portions of boiling ethanol, the mixture being allowed to boil each time for a few minutes. The combined ethanolic extracts, representing a total of 52 mg. of total P, were filtered while still hot, and treated under constant stirring with 0·6 g. of cadmium chloride dissolved in 0·8 ml. of water and diluted with 20 ml. of ethanol; a precipitate began to form immediately. The mixture was left standing for a few hours first at room temp., and then at 0°, overnight. The slightly yellowish and mostly amorphous precipitate was separated by centrifuging, and washed with ethanol. After draining off all remaining ethanol, the precipitate was dissolved in 8 ml. of water, and to the aqueous solution 95% (v/v) ethanol was added gradually until crystallization just began. After 5 days (at 0°), the snow-white crystalline material was collected in a sintered-glass crucible, washed with cold 80% (v/v) ethanol and dried in air on filter paper to constant weight.

Glycerol determination. With protein-free filtrates from samples deproteinized with $Ba(OH)_2$ and $ZnSO_4$, glycerol was estimated by the colorimetric periodate method of Lambert & Neish (1950) as modified by Ryley (1955). In filtrates from semen, fructose, which interferes with determinations of glycerol, was removed by the copper–lime precipitation technique, but this procedure involved small losses of glycerol owing to adsorption upon the copper–lime precipitate. GPC, analysed directly by the periodate method, gives about one-half the colour of the equivalent amount of free glycerol. An additional means of distinguishing between free and bound glycerol was obtained through chromatographic analysis with the procedure of Trevelyan, Procter & Harrison (1950).

Other determinations. Phosphorus was estimated by the method of Berenblum & Chain (1938), after the organic P-compounds had been converted into orthophosphate by

oxidation with perchloric acid. Fatty acid ester bonds were determined by the procedure of Stern & Shapiro (1953) with triacetin as a standard. Water-soluble ninhydrin-reacting N was measured by the method of Moore & Stein (1948), with the precipitation procedure described by Dawson (1956*a*). The O_2 uptake was measured manometrically at 37°.

Chemicals. Ovolecithin was prepared on an alumina column by the method of Hanahan (1954). Any acid-soluble impurities which might have been present were removed by washing with 0·05 M-$MgCl_2$ solution according to the procedure of Folch, Ascoli, Lees, Meath & LeBaron (1951). Lysolecithin was prepared enzymically with cobra venom (Hanahan, Rodbell & Turner, 1954). L-α-Glycerylphosphorylcholine was prepared by the method of Dawson (1956*b*), phosphorylcholine by the method of Plimmer & Burch (1937) and phosphorylethanolamine was synthesized by the procedure of Outhouse (1937).

Gifts of L-α-glycerylphosphorylethanolamine (Professor E. Baer), phosphatidylethanolamine (Drs Lea and Rhodes) and Indian cobra venom (Zoological Society of London) are gratefully acknowledged.

RESULTS

GPC and phosphorylcholine in semen and accessory gland secretions

All semen samples and accessory gland secretions obtained were examined for water-soluble phosphate esters by two-dimensional paper chromatography as described in the Experimental section. In every sample the major portion of the water-soluble P visible on the chromatogram could be accounted for as either GPC or phosphorylcholine, or both. Figs. 1*a* and *b* show typical examples of this, being chromatograms prepared from human and ram semen respectively. The amount of GPC and phosphorylcholine present in the semen varied considerably between the species, a roughly comparative assessment of the quantity as judged from the size and intensity of the chromatographic spots and the quantity of material used being given in Table 1. It is seen that the semen of the ram, goat, boar, stallion and rabbit contained exclusively GPC, whereas in man there was a small amount of GPC but a much larger quantity of phosphorylcholine. Our results concerning human semen are in agreement with those of Lundquist (1947*a*, *b*), who was the first to isolate phosphorylcholine from fresh human semen; they do not support the possibility of phosphorylcholine arising from GPC during the isolation procedure (Diament, 1955). Phosphorylcholine was also observed by us in small amounts in bull and monkey vesicular secretions, hedgehog accessory secretions and cock semen. In the boar and in the bull there was a particularly large concentration of GPC in the epididymal secretion, much larger than in any other secretion of the same species. The high content of GPC in the epididymal secretion might be taken to indicate that either the epididymis or the testis of the boar and bull is concerned with the production of seminal GPC. Because of this possibility, a few preliminary experiments were carried out with these two organs from a boar, and evidence was obtained that both contain phospholipase *B*, an enzyme capable of producing GPC from lysolecithin. Whether, however, this activity accounts for the formation of GPC under conditions *in vivo* remains to be ascertained.

The values for the GPC concentration, determined as acid-labile choline (Table 1), agree well with the comparative visual estimates obtained by chromatographic means. Although the iodide method for estimating choline is much more sensitive than the reineckate method it is rather unspecific, and consequently the values obtained with it have to be accepted with some reserve. Nevertheless, when GPC was determined in two samples of ram semen by the reineckate-precipitation method, the values were found to correspond closely with those obtained by iodide precipitation.

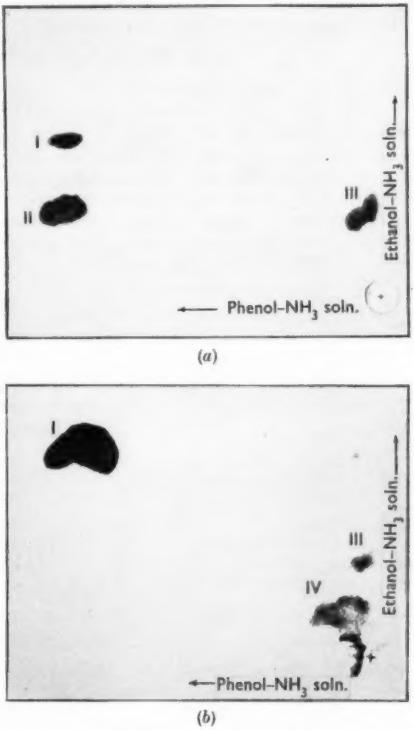

Fig. 1. Paper chromatograms prepared from the aqueous extracts of (*a*) human semen, (*b*) ram seminal plasma. I, Glycerylphosphorylcholine; II, phosphorylcholine; III, probably a nucleotide; IV, inorganic phosphate.

Moreover, in ram semen, which chromatographically shows very little acid-soluble P apart from GPC, the labile-choline values were in good agreement with the estimates of total acid-soluble P.

Glycerylphosphorylethanolamine, which accompanies GPC in nearly all mammalian tissues (Campbell & Work, 1952; Ansell & Norman, 1953; Dawson, 1955), was detected only occasionally on chromatograms, and then only in traces, amounting to less than 1% of the GPC content (Table 1). Phosphorylethanolamine was not detected in any of the specimens.

In view of the chromatographic and analytical results reported above, which showed that in several species, and particularly in the ram, GPC accounts for practically all the bound choline and acid-soluble P in seminal plasma, an attempt was made to isolate pure GPC from ram semen. The method used for this purpose was based on the procedure described by Baer & Kates (1948) for the preparation of the crystalline cadmium salt of synthetic GPC; it is described in the Experimental section. The crystalline cadmium salt of glycerylphosphorylcholine isolated from 40 ml. of ram seminal plasma weighed 470 mg. It was free from free choline and inorganic phosphate, and contained 23·3% of acid-hydrolysable bound choline; calc. for $(C_8H_{22}O_7NP)(CdCl_2),2H_2O$: 24·5% bound choline; the total phosphorus content was 6·00% P; calc.: 6·27%. Further proof concerning the purity of the substance isolated from ram seminal plasma was obtained by hydrolysing a sample for 20 min. at 100° in N-HCl. This resulted in complete hydrolysis to free choline and phosphoglycerol, which was shown chromatographically to be the sole P compound. No free fatty acids were liberated by hydrolysis. The small impurity present in the preparation of the cadmium salt of seminal GPC appeared to contain glutamic acid; no other amino acids were detected chromatographically, and the preparation was also free from carbohydrate, citric acid and phosphorylcholine.

Table 1. *Concentration of GPC, phosphorylcholine and glycerylphosphorylethanolamine in the semen and accessory-gland secretions of various species*

		GPC		Phosphorylcholine		Glyceryl-phosphoryl-ethanolamine.
Species	Material	Chromato-graphic determination	Acid-labile choline (mg. GPC/100 ml.)	Chromato-graphic determination	Choline released on incubation at 37° (mg. phosphoryl-choline/100 ml.)	Chromato-graphic determination
Ram	Semen	+ + + + +	1185, 1362, 1942	0	—	Trace seen in one sample
	Seminal plasma	+ + + + +	1601, 2040, 1770	0	—	—
Bull	Semen	+ + +	460, 237	Trace seen in one sample	—	Trace
	Seminal plasma	+ + +	496, 420, 375, 352, 110*	0	—	0
	Vesicular secretion	Trace	—	+ (2 samples)	—	0
	Epididymal secretion	+ + + + +	1490*	Trace	—	0
	Ampullar fluid	+ +	94*	Trace	—	0
Goat	Semen	+ + + + +	1382, 1550	0	—	Trace
Boar	Seminal plasma	+ +	108, 235	0	—	Trace
	Vesicular secretion	+ +	190	0	—	Trace
	Epididymal secretion	+ + + + + +	3060	0	—	Trace
Stallion	Semen	+	38, 113	0	—	Trace
	Ampullar secretion	+	120	0	—	0
Man	Semen	+	59, 59, 54, 90	+ + +	301, 286, 256, 380	0
Rat	Vesicular secretion	+ + +	654	Trace	—	0
Rabbit	Semen	+ +	215, 264, 370	0	—	0
Hedgehog	Secretion of 'prostate I and II'	+ +	—	+ +	—	0
	Secretion of 'prostate III'	0	—	+ + +	—	0
Monkey	Vesicular secretion	0	—	+ +	—	0
Cock	Semen	0	—	+	—	0

* Samples from the same bull.

Metabolic behaviour of GPC and phosphorylcholine

In view of the large amount of GPC detected in ram semen and seminal plasma it was of interest to investigate whether ram spermatozoa themselves could either produce or metabolize GPC. Washed suspensions of spermatozoa were therefore incubated for 3 hr. at 37°, aerobically or anaerobically, but no liberation of GPC, inorganic P or free choline was observed. Similarly, on incubating the washed sperm suspensions with added GPC there was no disappearance of the substrate or significant production of free choline or inorganic P. The same result was obtained when whole ram semen was incubated, either as such or after diluting it with P-free Ringer solution or with the phosphate diluent. The incubation of ram seminal plasma by itself had, again, no effect on the content of GPC, and it did not result in the appearance of free choline, glycerol or inorganic phosphate.

The semen and seminal plasma of the bull, like those of the ram, were inactive towards GPC. In three experiments in which bull semen or seminal plasma was incubated for periods of 3–24 hr., aerobically or anaerobically, with or without penicillin, there was no liberation of any appreciable quantities of free choline, glycerol or inorganic P, nor was there a disappearance of GPC (Table 2). This finding contrasts with the observation by Lundquist (1947*a*, *b*) that choline and inorganic P appear in bull seminal plasma on incubation.

Lundquist has demonstrated that, in human semen, phosphorylcholine breaks down rapidly to choline and inorganic phosphate within a short time after ejaculation, owing to the action of the powerful acid (prostatic) phosphatase which comes in contact with the substrate at the time of ejaculation. This observation has been fully confirmed in the present study. When fresh human semen was incubated for 90 min. at 37°, phosphorylcholine disappeared completely, and at the same time, equivalent amounts of free choline and inorganic phosphate were formed. The ease with which phosphorylcholine breaks down was even more evident in an experiment where freshly collected human semen was stored for 7 days at −15° instead of −78°. This period of storage was sufficient to cause the complete breakdown of the phosphorylcholine in the semen. Phosphorylcholine added to human seminal plasma also undergoes a rapid dephosphorylation; its complete disappearance was observed, for instance, in an experiment in which 0·5 ml. of a solution containing 6 mg. of sodium phosphorylcholine was incubated for 2 hr. at 37°, with 0·1 ml. of human seminal plasma and 0·4 ml. of veronal buffer at pH 5·7, 7 and 8·5 respectively. The ability of human seminal plasma to dephosphorylate phosphorylcholine exceeds markedly that of ram seminal plasma and even more so that of the bull; this behaviour is in agreement with the known acid phosphatase activity levels in the semen of these three species.

Apart from the large quantity of phosphorylcholine, human semen contains some GPC, as was mentioned before (Table 1). Unlike phosphorylcholine, however, GPC does not undergo the same rapid breakdown on incubation, and it can be detected chromatographically even after prolonged incubation of human semen (6 hr. at 37°). The difference between phosphorylcholine and GPC becomes even more pronounced when one compares the behaviour of the two substrates after they have been added as pure substances to the seminal plasma; incubation for 2 hr. at 37°, which as stated

Table 2. *Effect of incubation at 37° on the content of free choline and inorganic phosphate in bull and ram semen*

Sample	Conditions of incubation	Free choline (mg./100 ml.)	Inorganic P (mg./100 ml.)
Bull semen	No incubation	24	—
	3 hr., aerobic	28	—
	3 hr., anaerobic	28	—
Bull semen	No incubation	24	—
	3 hr., aerobic	26	—
	3 hr., anaerobic	36	—
Bull seminal plasma	No incubation	21	3
	18 hr., aerobic with penicillin	19	4
	18 hr., aerobic	24	3
Bull semen	No incubation	47	—
	24 hr., aerobic with penicillin	47	—
	24 hr., aerobic	46	—
Ram semen	No incubation	65	17
	3 hr., anaerobic	68	19
Ram semen diluted with P-free Ringer	No incubation	47	20
	3 hr., aerobic	53	32

previously hydrolyses phosphorylcholine completely, caused no breakdown of an added equivalent quantity of GPC. Thus the human seminal plasma appears to be devoid of any GPC diesterase, an enzyme which is distributed widely in mammalian tissues (Dawson, 1956*b*). The fact that acid phosphatase by itself is unable to attack GPC has been stated also by Schmidt, Bessman & Thannhauser (1953).

Effect of lecithin and lysolecithin on spermatozoa

In view of the close relationship of GPC to lecithin and lysolecithin, and the recent work on the 'protective' action of lecithin and related lipids on the sperm cells (e.g. Mayer, 1955), a series of experiments was carried out in order to compare the metabolic behaviour of GPC with that of lecithin and lysolecithin.

When pure lecithin, emulsified in Ringer solution, was incubated aerobically with a suspension of washed ram spermatozoa, there was sometimes, but not always, a considerable increase in O_2 utilization, which exceeded the control values by 40–380%. Choline, GPC, phosphatidylethanolamine and triolein did not produce a similar effect on the respiration, and as the lecithin had been carefully purified from acid-soluble impurities such as amino acids, the effect must be considered as a specific stimulation by lecithin. However, the increased O_2 consumption brought about by lecithin was not associated with the liberation of free choline or GPC, or phosphorylcholine; and it did not result in any decrease in the number of fatty acid ester bonds in the lecithin itself. It would thus appear that the increased O_2 consumption was not due to the enzymic hydrolysis of ester bonds in the lecithin and the subsequent metabolic oxidation of the hydrolytic products. Similar stimulatory effects of lecithin, not involving any apparent oxidation of lecithin, have been observed in studies on the respiration of liver mitochondria (Rodbell & Hanahan, 1955; Marinetti & Stotz, 1956), but in these experiments similar effects were obtained with phosphatidylethanolamine and lysolecithin. In the present study with ram spermatozoa, phosphatidylethanolamine neither increased the respiration nor was it metabolized by the sperm cells to produce ethanolamine, phosphorylethanolamine or glycerylphosphorylethanolamine.

Lysolecithin, on the other hand, had a marked inhibitory action on the respiration of washed ram spermatozoa, even after the lysolecithin preparation had been autoclaved sufficiently to destroy the enzymic activity of any residual cobra venom which might have been left during its preparation. This decrease in respiration was associated with a loss of motility as well as with a definite morphological change in the sperm cells, namely, a detachment of the sperm head from the midpiece–tail. Cobra venom itself, which, as is well known, contains an active phospholipase *A*, diminished the endogenous respiration of ram spermatozoa; although no acid-soluble P was liberated, there was a decrease in the fatty acid ester bonds of the sperm suspensions, indicating that the venom had probably attacked the lipid coating of the sperm cells. It is interesting to note in this connexion that the respiratory enzyme systems of isolated liver-mitochondria preparations are also known to be sensitive to a short incubation with phospholipase-containing venoms (Nygaard & Sumner, 1953; Nygaard, Dianzani & Bahr, 1954).

When human semen was incubated with lecithin for 2–7 hr. at 37°, at three different pH values, namely, 5·7, 7 and 8·5, no liberation of any acid-soluble P compounds was observed, above that found in the controls. It was thus not possible to confirm the claim of Cuzzocrea (1955) that an active phospholipase *C* is present in human semen.

DISCUSSION

It has been recognized for some time that mammalian seminal plasma is characterized by a high content of a phosphorus compound soluble both in trichloroacetic acid and ethanol, which does not readily liberate orthophosphate on acid hydrolysis. The similarity of this compound to glycerylphosphorylcholine was first stressed by Kahane & Lévy (1938, 1945, 1949), but it was only in 1952 that the French investigators actually accomplished the isolation of glycerylphosphorylcholine, as a ferric salt, from the seminal vesicle secretion of rats (Diament *et al.* 1952). A similar result was obtained by Lundquist (1953), who studied the vesicular secretions of rat, guinea pig and rabbit. The Danish author also succeeded, a few years earlier, in isolating from human seminal plasma another P compound that was difficult to hydrolyse and in identifying it as phosphorylcholine (Lundquist, 1947*a*, *b*). This finding, however, was criticized by the French investigators, who felt that the chemical method employed for the isolation of phosphorylcholine from human semen was so drastic that it could have caused a breakdown of glycerylphosphorylcholine to phosphorylcholine.

Our own results show that in certain species, notably man, phosphorylcholine is undoubtedly a natural, major constituent of the seminal plasma. Glycerylphosphorylcholine, however, also occurs in human semen but at a much lower concentration. In the bull, ram, goat, boar and stallion, on the other hand, the bulk of the 'bound choline' and 'difficultly-hydrolysable P' of seminal plasma was identified as glycerylphosphorylcholine, to the almost complete exclusion of phosphorylcholine.

The two phosphorus-containing compounds differ characteristically in their behaviour towards seminal phosphatase. Whereas phosphorylcholine is broken down readily immediately after ejaculation, giving rise to free choline and inorganic phosphate, glycerylphosphorylcholine is stable and does not break down appreciably. Although glycerylphosphorylcholine and phosphorylcholine accounted together for the major portion of the water-soluble organically bound phosphate in the seminal plasma of all species examined, chromatographic evidence was obtained for the existence in certain species of small amounts of some other P compounds. It is interesting to note, however, that glycerylphosphorylethanolamine, which accompanies glycerylphosphorylcholine in most mammalian tissues, is detectable in seminal plasma in trace amounts only.

The occurrence of phosphorylcholine and glycerylphosphorylcholine in the seminal plasma naturally raises the problem of the mechanism of their formation in the male accessory glands of reproduction. A possibility which merits particular attention is that these compounds may be derived from the metabolism of phospholipids in organs such as the epididymis and seminal vesicle.

SUMMARY

1. Glycerylphosphorylcholine and phosphorylcholine were found to account for the bulk of 'water-soluble bound choline' and 'acid-soluble, difficultly-hydrolysable phosphorus' in the seminal plasma of various mammals. In man, the content of phosphorylcholine exceeded considerably that of glycerylphosphorylcholine, whereas in ram, bull, boar, goat, stallion and rabbit practically the whole of bound choline was accounted for as glycerylphosphorylcholine. Bull and boar possess a particularly rich source of glycerylphosphorylcholine in the epididymis. Cock semen contained a small quantity of phosphorylcholine but no glycerylphosphorylcholine.

2. In addition to chromatographic means, glycerylphosphorylcholine was also identified by chemical isolation; 470 mg. of the crystalline cadmium salt was obtained from 40 ml. of ram seminal plasma. Glycerylphosphorylethanolamine was detected in trace amounts in some samples of seminal plasma, but phosphorylethanolamine was altogether absent.

3. Phosphorylcholine was readily dephosphorylated in semen after ejaculation. Glycerylphosphorylcholine was neither dephosphorylated nor changed in any other way by spermatozoa. Lecithin usually stimulated the oxygen uptake of spermatozoa, but this was not associated with an enzymic hydrolysis of ester bonds or the liberation of acid-soluble metabolic products. Lysolecithin, on the other hand, had a marked inhibitory effect on sperm respiration and motility.

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Glycerol Metabolism by Spermatozoa

By T. MANN and I. G. WHITE
A.R.C. Unit of Reproductive Physiology and Biochemistry, Molteno Institute, and Department of Veterinary Clinical Studies, University of Cambridge

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Although the inclusion of glycerol in media for freezing and long-term storage of bull semen is now a generally established practice, very little has been known hitherto about the mechanism underlying the uptake and metabolism of glycerol by spermatozoa. Recently, however, it has been shown by White, Blackshaw & Emmens (1954) that although glycerol does not affect the motility and respiration of spermatozoa in a fructose-containing medium, it increases markedly the oxygen uptake of washed ram- and bull-sperm suspensions, giving rise under certain conditions to an accumulation of lactic acid. Evidence was also brought forward recently by O'Dell, Flipse & Almquist (1956) that [^{14}C]glycerol is taken up readily and utilized by bovine spermatozoa, with the production of $^{14}CO_2$, and that the rate of glycerol uptake is higher in the absence than in the presence of seminal plasma. Further experiments on glycerol metabolism by ram spermatozoa are described in the present study, together with new observations on the metabolic behaviour of certain related substrates, and, particularly, phosphoglycerol, dihydroxyacetone and sorbitol (preliminary communication, Mann & White, 1956). These findings are discussed in the light of another recently completed study (Dawson, Mann & White, 1957), which describes the occurrence in ram seminal plasma of large quantities of bound glycerol in the form of glycerylphosphorylcholine.

MATERIAL AND METHODS

Semen. This was collected from ten rams by means of an artificial vagina and pooled for each experiment. Washed sperm suspensions were made by diluting 1·0–1·5 ml. of semen to 10 ml., in a tapered centrifuge tube, with one of the diluents listed below, centrifuging at about 1000 *g*, and washing the spermatozoa twice with the same amount of fresh diluent. The diluents were (i) the 'phosphate diluent' of White *et al.* (1954), containing 0·032 M-NaH_2PO_4,H_2O, 0·048 M-$Na_2HPO_4,12H_2O$, 0·040 M-NaCl and 0·004 M-KCl, and (ii) the calcium-free 'Ringer solution for spermatozoa' (Mann, 1946); in some experiments phosphate was left out from the Ringer solution ('P-free Ringer solution'), and in some other experiments each 100 ml. of the Ringer solution was supplemented further with 20 ml. of 0·25 M phosphate buffer, pH 7·4, prepared by mixing 190 ml. of an aq. soln. of 7·1 g. of Na_2HPO_4 (or 17·9 g. of $Na_2HPO_4,12H_2O$) with 10 ml. of N-HCl ('Ringer-phosphate solution').

Determinations. Glycerol was determined in protein-free filtrates from samples deproteinized with $Ba(OH)_2$ and $ZnSO_4$, by the method of Lambert & Neish (1950) as modified by Ryley (1955), and in some cases the identification of glycerol was carried out also by chromatography by the method of Trevelyan, Procter & Harrison (1950). The methods used for the determination of fructose, lactic acid, phosphorus, sperm respiration (in air, at 37°) and fructolysis (in N_2, at 37°) were as described by Mann (1946). The chromatographic identification of fructose was carried out by the method of Jermyn & Isherwood (1949).

RESULTS

Aerobic formation of lactic acid from glycerol by spermatozoa

The rate at which glycerol (0·01 M) is utilized by washed ram spermatozoa at 37° was determined in nine experiments, a fresh sperm suspension in 'phosphate diluent' being used each time, with about 3×10^8 cells/ml. The disappearance of glycerol under these conditions was nearly linear over the 3 hr. experimental period. The rate of disappearance was 0·5–1 mg. of glycerol/10^9 sperm/hr., with an average of 0·8 mg. Throughout the period of 3 hr., the utilization of glycerol was accompanied by an increase in O_2 uptake of the order of 0·1–0·25 c.c. of $O_2/10^9$ sperm/hr. over that of the glycerol-free control sample. The disappearance of glycerol was accompanied by the accumulation of lactic acid, at a fairly linear rate of 0·2–0·6 mg./10^9 sperm/hr. Assuming that one atom of oxygen was required for

the conversion of each molecule of glycerol into lactic acid ($C_3H_8O_3 + O \rightarrow C_3H_6O_3 + H_2O$), and assuming further that the excess of observed O_2 uptake was due to the oxidation of lactic acid ($C_3H_6O_3 + 3O_2 \rightarrow 3CO_2 + 3H_2O$), it can be calculated that between one-half and three-quarters of metabolized glycerol is accounted for by the oxidative breakdown.

When these experiments were repeated with, instead of the 'phosphate diluent', either a Ringer solution, which had a much lower P content, or a 'P-free Ringer solution', the ratio between glycerol disappearance on one side, and the O_2 uptake and lactic acid formation on the other, was found to be entirely different. In the P-poor or P-free medium, the rate of O_2 uptake was much higher, whereas that of lactic acid accumulation was found to be lower (Table 1).

These observations suggested that the high rate of lactic acid accumulation in the presence of phosphate may be due not to a higher rate of glycerol conversion into lactic acid but to a depressing effect of phosphate on the oxidative disposal of lactic acid by spermatozoa. This assumption was borne out fully by experiments in which the rate of lactate oxidation in the presence of phosphate was compared with that in a phosphate-free medium. As shown in Fig. 1, washed ram spermatozoa to which lactate has been added consumed O_2 at a lower rate in the presence than in the absence of phosphate. The depression in O_2 uptake brought about by the presence of phosphate corresponded also to a decreased disappearance of lactic acid, as determined chemically.

The metabolism of glycerol depends strictly on the presence of motile, i.e. fully active, spermatozoa. It can be abolished completely by heating the sperm suspensions at 70° for 2 min., and it can also be arrested promptly by the addition of spermicidal agents such as cetyltrimethylammonium bromide (Table 2). In this respect the process of glycerol oxidation resembles that of fructolysis, which is also extremely sensitive to spermicidal agents, particularly those which belong to the group of surface-active substances (Mann, 1954). Like the breakdown of fructose, that of glycerol appeared to be unaffected by the presence of antibiotics such as penicillin; no adverse effect was observed even after the addition of 1000 penicillin units/ml. of sperm suspension. Whereas, however, fructolysis can proceed in spermatozoa both anaerobically and aerobically, the process of glycerol utilization

Table 1. *Effect of phosphate on lactic acid accumulation and oxygen uptake of spermatozoa respiring in the presence of glycerol*

Each manometer flask contained 3·6 ml. of sperm suspension, 3×10^8 cells/ml.; the contents of each flask were analysed after 3 hr. incubation in air, at 37°.

Medium	Lactic acid (mg./3 hr.)	O_2 uptake (μl./3 hr.)
Phosphate diluent, no glycerol	0	178
Phosphate diluent + 0·01 M glycerol	1·05	459
P-free Ringer soln., no glycerol	0	224
P-free Ringer soln., + 0·01 M glycerol	0·15	705

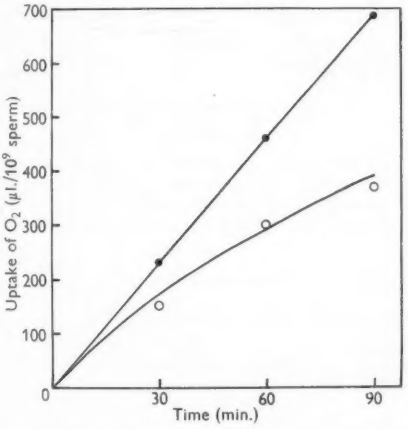

Fig. 1. Effect of phosphate on the oxidation of sodium lactate (0·01 M) by washed ram-sperm suspensions, 10^9 cells/3·3 ml., in air, at 37°. ●, With P-free Ringer as suspending medium for spermatozoa; ○, with the 'phosphate diluent' (0·08 M phosphate) as the medium.

Table 2. *Effect of cetyltrimethylammonium bromide (CTAB) on the utilization of glycerol by washed ram spermatozoa*

Glycerol, 0·01 M; incubation for 3 hr., in air, at 37°, with 2·2 ml. of a suspension of 'normal sperm' and of 'CTAB-treated sperm'. 'Normal sperm' suspension was prepared by mixing 1·5 ml. of ram semen with 1·5 ml. of phosphate diluent, leaving the mixture standing at 37° for 10 min. before centrifuging, and then washing it twice with 10 ml. of fresh phosphate diluent. 'CTAB-treated sperm' suspension was prepared by mixing 1·5 ml. of ram semen with 1·5 ml. of 0·005 M cetyltrimethylammonium bromide (CTAB), leaving the mixture standing at 37° for 10 min. before centrifuging, and then washing it twice with 10 ml. of fresh phosphate diluent.

	Glycerol utilized (mg./3 hr.)	O_2 uptake (μl./3 hr.)
Normal sperm + glycerol	1·83	381
CTAB-treated sperm + glycerol	0	16

depends strictly on the presence of O_2 and does not proceed in washed sperm suspensions incubated in the presence of N_2 instead of air. The essentially aerobic character of glycerol metabolism by spermatozoa is further reflected in the observation that in the presence of both fructose and glycerol a definite 'sparing effect' was exerted by glycerol on the rate of fructolysis, provided, however, that O_2 was also present; the rate of anaerobic fructolysis, unlike that of the aerobic fructolysis, remained unaffected in the presence of glycerol (Table 3). Although purely aerobic, the process of glycerol oxidation by living spermatozoa did not lead to the formation of hydrogen peroxide. All attempts to detect hydrogen peroxide by chemical means gave a consistently negative result, and the ratio between O_2 consumption and glycerol disappearance was found to be unaffected by the addition of catalase.

Oxidation of dihydroxyacetone

In the normal course of glycerol oxidation by washed spermatozoa, suspended either in a P-free Ringer solution or in the phosphate diluent, little or no accumulation of bisulphite-binding substances was observed. However, when such sperm suspensions were incubated in the presence of 0·02 M-NaF, particularly if glycerol was used in high concentrations (0·1 M), there was a marked increase in bisulphite-binding material, which accumulated after 3 hr. in amounts equivalent to 50–250 μg. of dihydroxyacetone. At the same time, fluoride had a strong inhibitory effect on the rate of glycerol utilization, O_2 uptake and lactic acid formation, as can be seen from Table 4, which also shows, for comparison, the effect of fluoride on fructose metabolism.

Over 50% of the bisulphite-binding material which accumulated in the aerobic incubation of spermatozoa with glycerol and fluoride was stable towards treatment with hot alkali, which suggests that at least some of it may have been pyruvic acid (Clift & Cook, 1932). No attempt has been made so far to identify the bisulphite-binding substances. It was found, however, that dihydroxyacetone added to washed sperm suspensions was oxidized as readily as glycerol, giving rise to an increased O_2 uptake and also to the formation of lactic acid in quantities as large as those which were produced from glycerol; the comparative effect of glycerol and dihydroxyacetone on the O_2 uptake of ram spermatozoa is shown in Fig. 2.

Metabolism of phosphoglycerol

Phosphoglycerol (both α- and β-) was observed to increase considerably the O_2 uptake of washed spermatozoa, though seldom to the same extent as

Table 3. *Effect of glycerol on aerobic and anaerobic sperm fructolysis*

Washed suspensions of 6×10^8 ram spermatozoa in 2 ml. of phosphate diluent were incubated for 3 hr. at 37°, in air and in nitrogen.

Additions to sperm	Fructose utilized (mg./3 hr.) Aerobically	Anaerobically
Fructose (0·005 M)	1·26	1·49
Fructose (0·005 M) + glycerol (0·010 M)	0·86	1·49

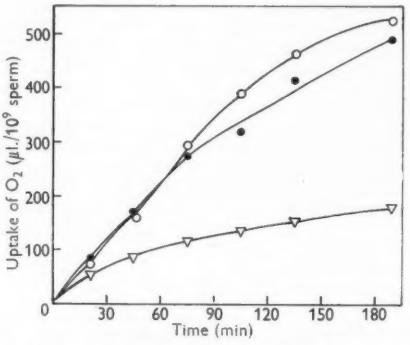

Fig. 2. Effect of glycerol (0·01 M) and dihydroxyacetone (0·01 M) on the oxygen uptake of sperm suspensions, 10^9 cells/3·3 ml. of phosphate diluent; in air, at 37°. ●, Glycerol; ○, dihydroxyacetone; ▽, no additions.

Table 4. *Effect of fluoride on the aerobic metabolism of glycerol and fructose by ram spermatozoa*

Washed sperm suspensions in 2·4 ml. of phosphate diluent were incubated for 3 hr. at 37°, in air.

Additions to the sperm suspension	Glycerol utilized (mg.)	Fructose utilized (mg.)	O_2 uptake (μl.)	Lactic acid formed (μg.)
None	—	—	124	0
Fluoride (0·02 M)	—	—	89	0
Glycerol (0·01 M)	1·75	—	415	990
Glycerol (0·01 M) + fluoride (0·02 M)	0·38	—	165	0
Fructose (0·005 M)	—	1·75	324	738
Fructose (0·005 M) + fluoride (0·02 M)	—	0·24	133	0

free glycerol. On closer analysis of this phenomenon, however, the conclusion was reached that the increased O_2 uptake was due, not to a direct oxidation of phosphoglycerol as such, but to the oxidation of free glycerol which had been set free from phosphoglycerol by dephosphorylation. It was found that phosphoglycerol was readily dephosphorylated by spermatozoa not only aerobically but anaerobically as well (Table 5); this dephosphorylation was accompanied by the liberation of glycerol. While, however, anaerobically, glycerol accumulated, aerobically it was oxidized, thus giving rise to an increase in the consumption of O_2. The effect of α- and β-phosphoglycerol, and that of free glycerol, on the O_2 uptake of spermatozoa is shown in Fig. 3.

Oxidation of sorbitol

Apart from glycerol, the effect of various other alcohols and related substances on the sperm respiration was tested, including ethanol, *n*-propanol, *iso*propanol, *n*-butanol, *iso*butanol, ethylene glycol, propylene glycol, inositol, mannitol, dulcitol, erythritol, glycidol (1:3-epoxypropan-1-ol), DL-glyceraldehyde and sorbitol. Of these substances, two, namely glycidol and DL-glyceraldehyde, had some depressing effect on the rate of O_2 uptake by washed spermatozoa, and sorbitol produced a marked increase in the O_2 consumption. It can be seen from Fig. 4 that the O_2 uptake of washed ram spermatozoa was increased by 0·1 M sorbitol to the same extent as by 0·01 M glycerol. The oxidation of sorbitol, like that of glycerol, was found to be accompanied by the formation of lactic acid and also, at the same time, by the appearance of a reducing sugar which gave the same ketose reactions as fructose and was yeast-fermentable; chromatographic analysis confirmed the identity of this sugar with fructose. The amounts of keto-sugar accumulating depended on the concentration in which sorbitol was used, and they were particularly large when sorbitol was added to spermatozoa simultaneously with glycerol. Thus in four experiments, the amounts of colorimetrically determined and yeast-fermentable ketose which accumulated at the end of a 3 hr. incubation period, in the presence of 10^9 sperm cells/3 ml. of phosphate diluent, were (in terms of fructose): 0–30 μg. from 0·01 M glycerol; 0–87 μg. from 0·1 M glycerol; 65–136 μg. from 0·01 M sorbitol; 430–970 μg. from 0·1 M sorbitol; and 570–1030 μg. from 0·1 M sorbitol + 0·01 M glycerol.

Table 5. *Dephosphorylation of phosphoglycerol by washed ram spermatozoa*

Incubation for 3 hr. at 37°; 3·4 ml. of sperm suspension (10^9 cells) in P-free Ringer solution. Each value represents an average from two experiments.

	Increase in inorganic phosphate (μg. of P)		Increase in the content of glycerol (μg.)
	Anaerobic	Aerobic	Anaerobic
α-Phosphoglycerol (0·01 M)	107	177	281
β-Phosphoglycerol (0·01 M)	63	150	240

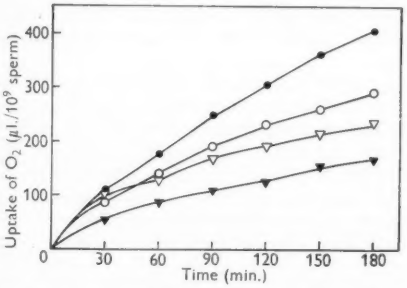

Fig. 3. Effect of α-phosphoglycerol, β-phosphoglycerol and glycerol on sperm respiration; 3·3 ml. of washed sperm suspension (10^9 cells) in phosphate diluent, incubated in air, at 37°. ●, Glycerol (0·01 M); ○, α-phosphoglycerol (0·01 M); ▽, β-phosphoglycerol (0·01 M); ▼, no additions.

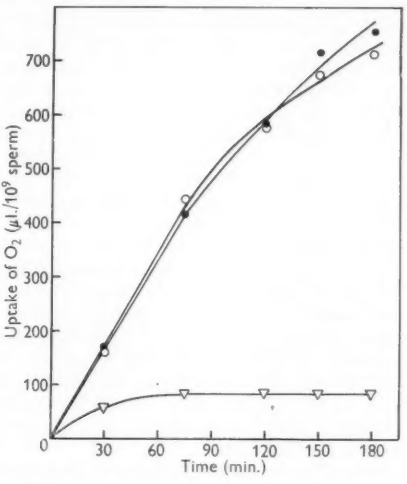

Fig. 4. Effect of sorbitol on sperm respiration; 3 ml. of suspension of washed ram spermatozoa (10^9 cells) in phosphate diluent, incubated in air, at 37°. ▽, No additions; ●, sorbitol (0·01 M); ○, glycerol (0·01 M).

DISCUSSION

In several mammalian species, including the ram, the seminal plasma is distinguished by a high content of glycerylphosphorylcholine; the concentration of glycerol bound in that form is of the order of 1 g./100 ml. of ram seminal plasma (Dawson *et al.* 1957). The evidence so far obtained, however, does not warrant the conclusion that this large reserve of bound glycerol can act either as a source of free glycerol or provide the sperm cells with a substrate for respiration. It is only after choline has been removed from glycerylphosphorylcholine by acid hydrolysis that the resulting product, phosphoglycerol, can be utilized by spermatozoa. It is shown in the present study that when phosphoglycerol is brought into contact with ram spermatozoa it is first dephosphorylated and then glycerol arising from this process is oxidized to lactic acid, probably via dihydroxyacetone. The exact mechanism underlying the oxidative dissimilation of glycerol by spermatozoa still remains to be explored in the light of the numerous recent studies on the various pathways of glycerol oxidation in other animal tissues and micro-organisms (Baranowski, 1949; Gunsalus & Umbreit, 1953; King & Cheldelin, 1952*a*, *b*; Asnis & Brodie, 1953; Magasanik, Brooke & Karibian, 1953; Wiame, Bourgeois & Lambion, 1954; Hauge, King & Cheldelin, 1955*a*, *b*). A possibility which merits particular attention is that the oxidation involves an initial dehydrogenation of glycerol to dihydroxyacetone, followed by a phosphorylative, glycolytic breakdown to lactic acid via phosphodihydroxyacetone. Another possibility is that the oxidation of dihydroxyacetone involves the 'phosphohexose shunt' and 'pentose cycle' as postulated recently by Hauge *et al.* (1955*b*) for *Acetobacter suboxydans*. The existence of a link between the oxidation of glycerol and that of sorbitol is another subject for future investigations. The oxidation of sorbitol to fructose by spermatozoa is probably due to sorbitol dehydrogenase, an enzyme analogous to that which occurs in the liver (Blakley, 1951; McCorkindale & Edson, 1954) and in male accessory organs (Williams-Ashman & Banks, 1954; Hers, 1955); fructose formed in that way would readily be broken down to lactic acid by the normal process of aerobic fructolysis. Such a mechanism would explain why glycerol, which itself is a lactic acid-producing substrate, should be able to exert a 'sparing effect' on the rate of aerobic fructolysis, thus facilitating the accumulation of fructose from sorbitol.

Although glycerol is metabolized aerobically by spermatozoa it seems unlikely that the preserving effect of glycerol on frozen sperm, described by Polge, Smith & Parkes (1949), is dependent on the metabolic activity of spermatozoa. The concentrations of glycerol which must be added to semen for freezing purposes are many times higher than those required for the respiratory activity of spermatozoa. Moreover, although glycerol is most effective in protecting spermatozoa from the harmful effects of freezing in certain animals such as cock, bull and herring, it appears to be much less effective in other species, including ram (White *et al.* 1954). A much more likely explanation is that glycerol exerts its action through a protective influence on sperm colloids, possibly owing to its high electrolyte- and water-binding capacity. As pointed out by Lovelock & Polge (1954), the damage suffered by spermatozoa during freezing and thawing could be explained by the exposure to excessive concentrations of salt after the removal of water as ice. The protective action of glycerol could thus be ascribed to its ability to prevent the salt concentration rising above a critical level. Another possibility which must be borne in mind is that glycerol may act as a 'stabilizing' agent on sperm proteins and enzymes; glycerol, as it is well known, combines the properties of a protein solvent with the ability to protect proteins from denaturation.

SUMMARY

1. Ram spermatozoa, separated from seminal plasma by centrifuging and washing, metabolize glycerol aerobically to lactic acid. The yield of lactic acid is higher in the presence than in the absence of phosphate owing to an inhibitory effect of phosphate on the oxidation of lactic acid by spermatozoa.

2. Dihydroxyacetone is oxidized by spermatozoa as rapidly as glycerol, giving rise to lactic acid.

3. Phosphoglycerol (both α- and β-) increases the respiration of spermatozoa. This is due to the oxidation of glycerol set free from phosphoglycerol by dephosphorylation. Glycerylphosphorylcholine, which is a natural constituent of ram semen, is neither dephosphorylated nor oxidized by spermatozoa.

4. Apart from glycerol a number of alcohols and related substances have been tested with respect to their effect on respiration. One of them, sorbitol, is shown to be oxidized rapidly by spermatozoa to fructose, which is then broken down to lactic acid.

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A Method for the Dermination of Aldosterone, Cortisol and Corticosterone in Biological Extracts, Particularly Applied to Human Urine

By P. J. AYRES, O. GARROD, SYLVIA A. SIMPSON* and J. F. TAIT*
Department of Physics applied to Medicine and the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, and the Institute for Clinical Research, Middlesex Hospital, London, W. 1

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Increased amounts of aldosterone have been found in the urine of patients with certain pathological conditions (Gaunt, Renzi & Chart, 1955), and its analysis in human urine and body fluids is therefore of some interest. Methods previously used for the estimation of this compound in biological extracts have been based on these procedures: (*a*) bioassay of crude (Liddle, Cornfield, Casper & Bartter, 1955; Liddle, Bartter, Duncan, Barber & Delea, 1955; Mader & Iseri, 1955; Gordon, 1955) or purified (Luetscher & Axelrad, 1954; Venning *et al.* 1955; Cope & Llaurado, 1954; Genest, 1955; Simpson & Tait, 1955*a*) extracts, partition-column chromatography being used for the separation of the steroids by Simpson & Tait and paper chromatography by the other workers; (*b*) separation in the propylene glycol–toluene system (Zaffaroni & Burton, 1951), followed by chromatography in the Bush C system (Bush, 1952), the resulting purified aldosterone being estimated by visual examination of the soda-fluorescent spot (Neher & Wettstein, 1955).

Both methods have serious disadvantages. The bioassay results can be influenced by the effects of administered impurities or, in some cases, by other corticoids (Luetscher & Curtis, 1955). The double-chromatography method, first used for the analysis of adrenal gland extracts (Simpson & Tait, 1953), has not yet been shown to be either specific or quantitative when relatively large amounts of other corticoids are present. Moreover, the procedure described by Neher & Wettstein (1955) gives low values for aldosterone in normal urine (see below).

The method described here depends on the extensive purification of aldosterone diacetate followed finally by an objective measurement of the fluorescence on a paper chromatogram. Specific measurements of cortisol and, if necessary, of corticosterone, can be made at the same time. It seems possible, by this method, to measure aldosterone in one-third of a 24 hr. specimen of normal urine with a precision and accuracy of 10–15 %. It has been applied to the analysis of adrenal homogenate, human urine and peripheral blood. From the stage immediately preceding the silica-gel column (Fig. 1), the method is basically the same for the analysis of urinary, blood or adrenal gland extracts. This paper is concerned only with its use with human urine. A preliminary account of some of this work has already been published (Ayres, Simpson & Tait, 1956).

* External scientific staff, Medical Research Council.

EXPERIMENTAL

Nomenclature

Aldosterone, cortisol, tetrahydrocortisone, tetrahydrocortisol and corticosterone are used as trivial names for 11β:21-dihydroxy-3:20-dioxo-pregn-4-en-18-al, 11β:17α:21-trihydroxypregn-4-ene-3:20-dione, 3α:17α:21-trihydroxypregnane-11:20-dione, 3α:11β:17α:21-tetrahydroxypregnan-20-one and 11β:21-dihydroxypregn-4-ene-3:20-dione respectively throughout this paper.

Materials

Radioactive steroids. [4-^{14}C]Cortisol and corticosterone (specific activity about 1·5 mc/m-mole), supplied by the U.S. Public Health Department, were purified by partition-column chromatography with the solvent systems described later in this paper. About 5% of the radioactivity was found in effluent fractions less polar than would be expected for the pure steroids; these fractions were discarded.

Aldosterone [*carboxy*-^{14}C]diacetate (specific activity about 1·4 mc/m-mole) was prepared as previously described (Avivi, Simpson, Tait & Whitehead, 1954).

Reagents and solvents

Kieselguhr. Celite 545 was used as the supporting medium in the partition columns. This was washed once with conc. HCl, then to neutrality with water and finally dried at 120° for 15 hr.

Silica gel. Purified silica gel (Davison Chemical Co., grade 950, 60–200 mesh) was used untreated. Although it has been reported that this gel can degrade steroids under certain conditions (A. S. Meyer, personal communication), various amounts (0·1–5 μg.) of aldosterone, cortisol and corticosterone have been chromatographed in the Bush & Sandberg (1953) system with over 95% recovery of unchanged steroid (Simpson & Tait, unpublished observations) with one untreated batch of silica gel.

Benzene (May and Baker Ltd.) was redistilled. Chloroform (May and Baker Ltd.) was redistilled over potassium carbonate after addition of ethanol (2% v/v). Methanol (James Burroughs) was redistilled over calcium. Ethyl acetate (B.D.H. Ltd.) was washed with 5% aqueous solution of sodium carbonate, dried, and freshly redistilled over potassium carbonate. Acetic anhydride (AnalaR) was used untreated. Pyridine (B.D.H. Ltd.) was redistilled weekly. Blue tetrazolium, 3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride (G. T. Gurr Ltd.). Light petroleum, boiling points 80–100° (May and Baker Ltd.).

Methods

The method employed is shown in Fig. 1.

Extraction. Unpreserved urine (1 l.) from a 24 hr. collection is acidified to pH 1 which, as first shown by Luetscher & Axelrad (1954), increases the yield of free aldosterone. An amount (2 μg.) of [4-^{14}C]cortisol and, if required, 1 μg. of [4-^{14}C]corticosterone, is added to the urine to enable the isotope-dilution method to be used for analysing these steroids. The urine is passed continuously through 500 ml. of chloroform for 24 hr. at room temperature, the Cohen-type extractor (Cohen, 1950) being used, and is then further extracted by shaking with 250 ml. of fresh chloroform. The chloroform phases are washed with 150 ml. of 0·05N-Na_2CO_3 and 100 ml. of water, which are both back-extracted with 100 ml. of chloroform. The combined chloroform extracts are taken to dryness *in vacuo* at 40° and further purified by passing through a 3 g. silica-gel column (Bush & Sandberg, 1953). The dried residue from the ethyl acetate–methanol eluate is chromatographed on a kieselguhr-partition column (Morris & Williams, 1953; Simpson & Tait, 1953).

Partition-column chromatography. A column 60 cm. long, of 1 cm. internal diameter, is packed with 26 g. of Celite 545, with water–methanol (1:1, v/v) as the stationary and benzene–ethyl acetate (5·2:1, v/v) as the mobile phase. The purified extract is added to the column in a total volume of 2 ml. of mobile phase containing about 50 μg. of Sudan

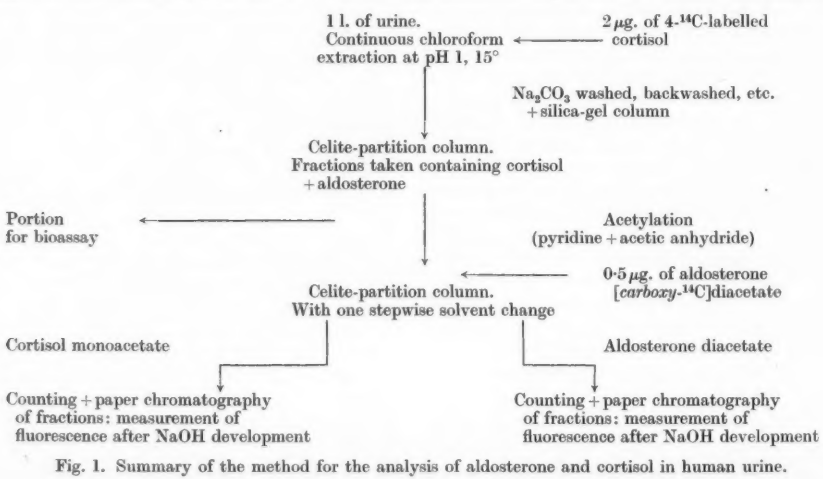
1 l. of urine.
Continuous chloroform extraction at pH 1, 15°
2 μg. of 4-14C-labelled cortisol
Na2CO3 washed, backwashed, etc.
+silica-gel column
Celite-partition column.
Fractions taken containing cortisol
+aldosterone
Portion for bioassay
Acetylation
(pyridine + acetic anhydride)
0·5 μg. of aldosterone
[carboxy-14C]diacetate
Celite-partition column.
With one stepwise solvent change
Cortisol monoacetate
Aldosterone diacetate
Counting + paper chromatography of fractions: measurement of fluorescence after NaOH development
Counting + paper chromatography of fractions: measurement of fluorescence after NaOH development

Fig. 1. Summary of the method for the analysis of aldosterone and cortisol in human urine.

red dye. Fractions (3 ml.) are collected. Aldosterone and cortisol have identical partition coefficients in this system and appear together consistently in the same fractions, i.e. fractions 6–12 inclusive from the appearance of red dye. These fractions are pooled, taken to dryness *in vacuo* and acetylated overnight with 0·15 ml. of acetic anhydride in 0·3 ml. of pyridine at room temperature. A portion for bioassay may be taken before acetylation. Aldosterone [*carboxy*-^{14}C]diacetate (0·5 μg.) is then added and the acetylated mixture dissolved in 2 ml. of mobile phase and run on a partition column 30 cm. long, of 1 cm. internal diameter, 13 g. of Celite 545 with methanol–water (4:1, v/v) being used as the stationary and light petroleum–benzene (3:2; v/v) as the mobile phase. When 12 × 5 ml. fractions have been collected after adding the steroids to the column, the mobile phase is changed to light petroleum–benzene (2:11; v/v), to hasten the appearance of the cortisol monoacetate. Portions of the fractions (20% for aldosterone diacetate, 10% for cortisol monoacetate) are evaporated to dryness and analysed for radioactivity. The dried residues are transferred with chloroform to the wells of cavity microscope slides, which are then placed as close as possible to a mica-window counter (G.E.C. type E.H.M. 2). This arrangement has an overall efficiency of about 10% and, hence, 1 μg. of [4-^{14}C]cortisol or 1 μg. of aldosterone [*carboxy*-^{14}C]-diacetate give approximately 850 counts/min., with a background of 10 counts/min. Correction for self-absorption is unnecessary for the counts obtained from the samples containing aldosterone diacetate. The presence, however, of solid material of unknown identity in some of the cortisol monoacetate fractions usually necessitates a self-absorption correction of about 10% of the initial count. This is carried out by eluting the sample from the slide after obtaining the initial count, adding 1 μg. of [4-^{14}C]cortisol and recounting. Fig. 2 shows the results for a typical analysis. Control experiments have shown that eluting and replating the sample reduces the count by less than 2%. All results in this paper have been obtained in this manner. Recently it has been found that, with a Tracerlab flow-counter type SC/16, samples can be counted after transfer with chloroform to a 2 cm. diameter circle of lens tissue mounted on a copper planchet. The count is then increased by a factor of 4·5 with a background of 23 counts/min., and a correction for self-absorption is unnecessary.

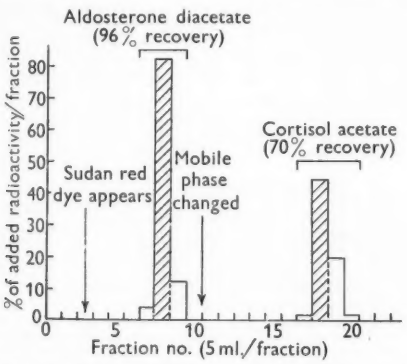

Fig. 2. Analysis of radioactive aldosterone diacetate and cortisol monoacetate in the eluates of a partition column. The radioactivity in the eluates, as measured by a mica-window Geiger counter, is expressed as a percentage of that of the cortisol added initially to the urine or of the aldosterone diacetate added to this column. Portions from the fractions (cross-hatched rectangles) containing peak amounts of each steroid are taken for paper chromatography.

Estimation of aldosterone diacetate and cortisol monoacetate. Portions of the peak radioactive fractions are chromatographed in paper against standards (0·5 and 1 μg. of aldosterone diacetate; 0·75 and 1·5 μg. of cortisol monoacetate) with systems (Bush, 1952) in which the derivatives run at an R_F value of about 0·5. The B 3 system is used for aldosterone diacetate and a modified B 2 system [methanol–water (4:1, v/v) stationary phase] for cortisol monoacetate. The paper is sprayed with a solution of 0·004% blue tetrazolium in 10% (w/v) aqueous NaOH (Bush, 1952), which allows a preliminary quantitative examination to be made of the resulting formazan. The soda fluorescence is developed by heating and the orange-yellow light emitted is measured quantitatively by a fluorimeter described in the Addendum. The specific activity of the steroids is obtained, from which the amounts initially present in the urine can be calculated. The values for cortisol are thus corrected for losses that have occurred throughout the entire procedure, those for aldosterone for losses after addition of the radioactive diacetate.

The fractions less polar than cortisol in the first Celite column are used for the estimation of corticosterone after running on a partition column, 60 cm. long, of 1 cm. internal diameter, methanol–water (4:1, v/v) being used as the stationary and benzene–light petroleum (10:1, v/v) as the mobile phase. The column is analysed for radioactivity as previously described. The fraction containing most radioactivity is divided into two portions which are run against standards of corticosterone in the same paper-chromatographic system as is used for cortisol monoacetate. The corticosterone initially present in the urine is now estimated in the same manner as described for cortisol.

Other compounds of biological interest, such as 11β- and 17α-hydroxyprogesterone and deoxycorticosterone, appear in the fractions less polar than corticosterone in this column. If such fractions are pooled, these three compounds can be analysed in the same column system as is used for the purification of aldosterone diacetate without changing the mobile phase.

These procedures for the analysis of steroids can also be applied without further modification to small-scale preparative work (less than 10 mg.).

RESULTS

In the course of analysing about thirty urine specimens by this method, the only soda-fluorescent spots which have been seen in the final paper chromatogram of the aldosterone diacetate were those running at the same speed as the standards. Occasionally, spots giving blue fluorescence or reacting with the tetrazolium salt have appeared, but not close enough to the aldosterone diacetate to interfere with the development or measurement of the yellow soda fluorescence. The final paper

chromatogram of the purified cortisol monoacetate has shown two positive soda-fluorescent spots in about one-fifth of the analyses. One which appeared in all chromatograms runs at the same speed as the standards of cortisol monoacetate, and gives an equivalent reaction with the tetrazolium salt. The other is more polar than cortisol monoacetate and gives a positive soda-fluorescent reaction only. It is, however, sufficiently separated from the cortisol monoacetate not to interfere when the soda fluorescence of this last compound is being measured. The tetrazolium and soda-fluorescence reactions also give equivalent results in the estimation of aldosterone diacetate and the reacting areas are superimposed symmetrically. For both aldosterone diacetate and cortisol monoacetate the amounts as measured by fluorescence or by the tetrazolium reaction are correlated with corresponding radioactive counts in successive fractions of the final column.

The final paper chromatogram of the purified corticosterone usually shows several spots in addition to that giving both positive tetrazolium and soda-fluorescence reactions and running at the same speed as the standards of corticosterone. One of these extra spots, usually present after fractionating normal urine, gives blue fluorescence on development of the chromatogram. As this compound runs close to corticosterone, it may add to the fluorescence measured from this last compound. Owing to the narrow transmission band of the secondary filters, this blue fluorescence is unlikely to contribute appreciably to the fluorimeter readings, as is indicated by very low values found for corticosterone in the patient with bilateral adrenalectomy (Table 3), when the interfering compound was still present in normal amounts. Nevertheless, because of this potential interference the corticosterone values quoted in this paper should be regarded as maximum estimates only.

Comparison of bioassay and physicochemical methods

As previously described, a portion of the pooled fractions containing aldosterone and cortisol from the first partition column may be taken for bioassay in the sodium/potassium test (Simpson & Tait, 1952). The bioassays were carried out as described previously except that radioactive sodium and potassium were not injected and the urinary sodium/potassium ratio was measured with a flame photometer (Warren, 1952). Log sodium/potassium was used as the response metameter and log (amount of injected material) as the dose metameter. Recent studies have shown this procedure to be preferable to that used in the original method (Jones, Simpson & Tait, 1956). Four-point assays were employed, sixteen rats being injected with the urinary extract and an equal number with pure aldosterone. In the amounts present, cortisol would have no effect on the urinary sodium/potassium excretion. Hence concomitant measurements for aldosterone may be made on the same urine specimen with the physicochemical and bioassay procedures. Fig. 3 shows five such pairs of values for specimens of five normal urines. Statistical analysis gives

$$y = 1{\cdot}07 - 0{\cdot}52x,$$

where $y \equiv$ aldosterone in μg. (bioassay) and $x \equiv$ aldosterone in μg. (physicochemical), and

$$\delta y = 1{\cdot}66, \quad \delta x = 1{\cdot}52,$$

where δy and δx are the standard errors of the estimate. Thus

$$\delta y = \Delta y \sqrt{(1 - r^2)}, \quad \delta x = \Delta x \sqrt{(1 - r^2)},$$

where $\Delta y \equiv$ variance of y, $\Delta x \equiv$ variance of x, the correlation coefficient (r) being $+0{\cdot}986$. There is thus no indication from these data that the values given by the two methods are not equivalent.

Recoveries

Specimens (24 hr.) of urine from male patients with no obvious endocrine dysfunction were used to investigate the recovery of non-radioactive cortisol and aldosterone and [4-^{14}C]corticosterone and cortisol. When necessary, the specimen was diluted to 2 l. with water, two portions of 1 l. being taken, each representing one-half or less of a 24 hr. urinary output. [4-^{14}C]Cortisol (2 μg.) was added to all the portions as for the routine procedure. The initial aldosterone and cortisol were estimated for one of

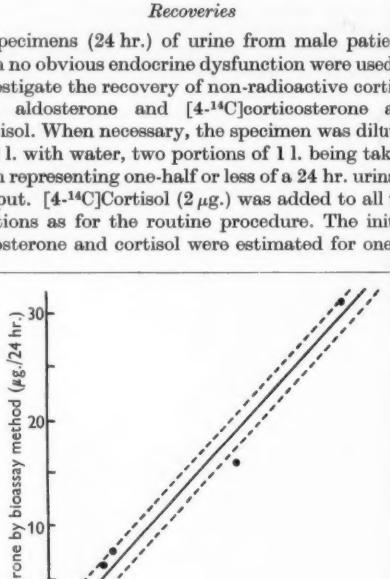

Fig. 3. Comparison of biological and physicochemical estimation of aldosterone on the same specimens of urine (5 male patients). The full line is the best-fitting line as calculated by the method of least squares. The vertical distance from the full line to the dotted line is equal to δy (standard error of the estimate), where $\delta y = \Delta y\sqrt{(1 - r^2)}$, Δy is the variance of the bioassay results and r ($= +0{\cdot}98$ for the five points) is the correlation coefficient.

Table 1. *Recovery of non-radioactive aldosterone and cortisol, and [4-^{14}C]cortisol and [4-^{14}C]corticosterone, after addition of them to the urine of male subjects*

Steroid added	No. of determinations	Mean recovery (%)	Standard error	Coefficient of variation (%) S.D. × 100 / Mean
Non-radioactive aldosterone (15 μg.)	10	77	±6	23
Non-radioactive cortisol (33 μg.)	7	97*	±5	12
[4-^{14}C]Cortisol (2 μg.)	25	70	±1·5	10
[4-^{14}C]Corticosterone (1 μg.)	7	73	±1	2·5

* Measured by isotope-dilution method.

the portions and 15 μg. of aldosterone or 33 μg. of non-radioactive cortisol or both were added to the other sample and the analysis was repeated. After correcting for the initial values, the recovery of the non-radioactive steroids could be calculated.

To each of seven specimens 1 μg. of [4-^{14}C]corticosterone was added as in the routine procedure. The recoveries of the two radioactive steroids, corticosterone and cortisol, were calculated from the radioactivity added to the urine compared with that present in the form of corticosterone or cortisol monoacetate in the fractions of the final partition column.

Table 1 shows the experimental results for all the recovery experiments. Any loss of non-radioactive aldosterone on the final partition column was corrected by applying the isotope-dilution method after adding radioactive aldosterone diacetate. In practice this loss was generally less than 5%. The value of 97 ± 5 (S.E.)% for the estimation of added non-radioactive cortisol obtained by the isotope-dilution method was already corrected for losses throughout the procedure. The actual recoveries of non-radioactive and radioactive cortisol were therefore not significantly different. The recovery values for radioactive cortisol and corticosterone and non-radioactive aldosterone did not differ significantly from one another. They were of the order of 70% from beginning to end of the procedure.

The mean initial values in the portions analysed were 4·0 ± 0·4 (S.E.) μg. of aldosterone and 13·1 ± 2·5 (S.E.) μg. of cortisol. Hence the amounts of non-radioactive steroid added were about three times those initially present. There was no significant difference in the recovery of 2 μg. of [4-^{14}C]cortisol whether the 33 μg. of non-radioactive cortisol was added or not. The procedure described should therefore give a reasonable estimate of recoveries of aldosterone released by acid treatment, and of free cortisol, when applied to normal urine.

Reproducibility

Two equal portions from the same specimen of urine, obtained as described for the recovery experiments, were analysed for cortisol and aldosterone to provide duplicate data. The results for the urine from four normal subjects are shown in Table 2.

Table 2. *Duplicate estimations of cortisol and aldosterone in urine samples from control male subjects*

Cortisol (μg./24 hr.) Values	Cortisol (μg./24 hr.) Mean	Aldosterone (μg./24 hr.) Values	Aldosterone (μg./24 hr.) Mean
86·2 / 76·6	81·4	14·6 / 13·2	13·9
31·9 / 27·8	29·8	18·6 / 14·1	16·4
39·4 / 40·0	39·7	22·2 / 24·8	23·5
28·9 / 33·6	31·2	4·3 / 6·7	5·5

Values for normal and pathological subjects

Table 3 shows the values for the 24 hr. urinary excretion of aldosterone, cortisol and corticosterone, as measured by this method in a series of male subjects with no obvious endocrine dysfunction. It also shows the value for a single patient after bilateral adrenalectomy who was maintained on a daily dose of 50 mg. of cortisone plus 0·25 mg. of fluorohydrocortisone, cortisone alone having failed to provide adequate electrolyte balance. Data are also included from two patients with primary aldosteronism or Conn's syndrome (the patient reported by Chalmers, FitzGerald, James & Scarborough, 1956, and another patient kindly provided by Dr J. R. Nassim of Stanmore Orthopaedic Hospital) and three patients with other pathological conditions.

Corticosterone and cortisol were measured concomitantly in urine specimens from seven normal male subjects. The values are included in Table 3. The mean ratio of cortisol to corticosterone was 7·4 with a range of 2·7–13·7; these should be considered as minimum values only, because of the possible lack of specificity of the method for the estimation of corticosterone.

Table 3. *Daily excretion of aldosterone, cortisol and corticosterone in a control series of male subjects and some patients with pathological conditions*

Subject	Aldosterone (μg./24 hr.)	Cortisol (μg./24 hr.)	Corticosterone (μg./24 hr.)
Bilateral adrenalectomy maintained on 50 mg. of cortisone + 0·25 mg. of fluorohydrocortisone	0·5	65	0·4
Primary aldosteronism (Chalmers *et al.* 1956):			
(*a*) Immediately before operation	28	21	—
(*b*) After removal of tumour	1	11	—
Primary aldosteronism (Dr J. R. Nassim, unpublished observations):			
(*a*) Immediately before operation	33·3	20·8	0·8
(*b*) After removal of tumour	0·8	11	—
Adrenal carcinoma causing virilization and severe hypertension:			
(*a*) Before operation	5·8	370	34
(*b*) After removal of tumour	15	5·3	4
Cushing's syndrome due to metastasizing adrenal carcinoma (post-adrenalectomy with recurrence of symptoms)	4·0	1200	—
Salt-losing nephritis (maintained in normal electrolyte balance)	127	42·8	3
Mean and range of control male subjects	11 (4·6–23·5) (31 subjects)	35 (13–86) (24 subjects)	5·8 (2·2–9·0) (7 subjects)

DISCUSSION

Of those hormones potent in prolonging the life of adrenalectomized animals, cortisol, on a weight basis, is probably the major one secreted by the normal human adrenal cortex. The main metabolites of this compound in human urine are tetrahydrocortisone and tetrahydrocortisol (Schneider, 1952; Burstein, Savard & Dorfman, 1953). The 20β-hydroxy and 17-oxo products of these two compounds are also present, and to a lesser extent, dihydrocortisone, 17α:21-dihydroxypregnane-3:11:20-trione (Fukushima *et al.* 1955; Dorfman, 1954; Schneider, 1952). Smaller amounts of metabolites retaining the original A-ring structure have been found, including 11β:17α:20β:21-tetrahydroxypregn-4-en-3-one (Reichstein's compound E), 17α:20:21-trihydroxypregn-4-ene-3:11-dione (Reichstein's compound U), cortisone and also unchanged cortisol (Holness, 1956; Burton, Zaffaroni & Keutmann, 1951). Aldosterone is secreted in much smaller quantities than cortisol by the mammalian adrenal cortex under normal physiological conditions. Indirect evidence suggests that the ratio of cortisol to aldosterone in normal human adrenal venous blood is of the order of 100:1 (Simpson & Tait, 1955*b*). This accounts for the present lack of information on metabolites of aldosterone. Tetrahydrocortisone and tetrahydrocortisol in human urine are conjugated with glucuronic acid, but the amounts of cortisone and cortisol extracted by chloroform are not increased by incubation of the urine with β-glucuronidase according to published reports (Gray & Lunnon, 1953), nor by mild acid hydrolysis, i.e. standing at pH 1 for 24 hr. at room temperature (Simpson & Tait, unpublished observations). The extractable free aldosterone, however, is increased by both these procedures, acid hydrolysis being the more productive according to most workers (Axelrad, Cates, Johnson & Luetscher, 1955; Venning *et al.* 1955; Mills, 1954; Neher & Wettstein, 1955). As measured by the method described in this paper, the yield of aldosterone is increased 20–30-fold when this is continuously extracted from urine at pH 1, compared with identical treatment at pH 6 (Simpson & Tait, unpublished observations). Presumably this treatment hydrolyses a conjugate, the nature of which has not yet been established. The urinary excretion of tetrahydrocortisone plus tetrahydrocortisol in normal subjects is of the order of 6 mg./day, the secretion of cortisol by the adrenal cortex being about 20 mg./day; that of aldosterone is probably of the order of 250 μg./day, this being the amount necessary to maintain patients with Addison's disease in adequate electrolyte balance (Mach, Fabre, Duckert, Borth & Ducommun, 1954). The maximal amount of metabolites of aldosterone excreted in the urine would therefore be expected to be 250 μg./day, and that of any tetrahydro products not more than 60 μg./day. The only known measurable metabolite of aldosterone would appear to be the presumed conjugate which, on acid hydrolysis, yields 10 μg. of free aldosterone/day. This should represent an appreciable fraction of the excretion products of this hormone, and it is doubtful whether the analytical procedure could be greatly simplified as a result of the characterization

of any tetrahydro derivatives of aldosterone. The measurement in peripheral blood presents an even greater problem since the concentration of the free compound appears to be 0·08 μg./100 ml. of whole blood (Simpson & Tait, 1955*b*), and this is not increased by acid hydrolysis at pH 1 (Simpson & Tait, unpublished observations). Since only small amounts of free cortisol are excreted in the urine compared with tetrahydrocortisone and tetrahydrocortisol, it is not yet known whether any useful purpose is served by the estimation of this compound when fairly simple methods are available for the specific measurement of the tetrahydro metabolites. However, until this aspect of the problem has been thoroughly investigated, it was thought worth while to incorporate a determination of free cortisol into the method, little extra labour being involved.

The achievement of complete specificity is of paramount importance in measuring quantities of steroid of the order of 10 μg./24 hr. The claim for specificity in determining aldosterone by the method just described is based on the following considerations:

(*a*) The separative methods used purify a compound which behaves like authentic aldosterone in a partition column having resolving power of approximately 400 theoretical plates. After acetylation, the amount of this material in the fractions of the final partition column, as measured by soda fluorescence after paper chromatography, can be correlated with the radioactivity of the same fractions. A small amount of authentic aldosterone [*carboxy*-^{14}C]diacetate is added to the sample before column chromatography. Paper chromatography of a portion of the fraction containing most radioactivity shows a single soda-fluorescent spot running at the same speed as authentic aldosterone diacetate.

(*b*) This spot gives symmetrical reducing and fluorescence reactions when developed with a solution of tetrazolium salt in sodium hydroxide. The two reactions are equivalent in terms of the standards.

(*c*) The amount of aldosterone as measured by the physicochemical technique agrees with the values obtained by concomitant bioassay of the purified free aldosterone before formation of the diacetate.

(*d*) The amount of aldosterone that could be detected in the urine of a bilaterally adrenalectomized patient who was fully maintained on cortisone was very low. The values could also be correlated with other clinical conditions characterized by disturbances in electrolyte metabolism but with normal excretion of cortisol or its metabolites.

(*e*) As was also found by other workers, with different methods, the yield of free aldosterone is greatly increased by acid hydrolysis at pH 1, whereas that of free cortisol is not.

The precision of the measurement of aldosterone and free cortisol can be estimated from the data on the recovery and duplicate experiments shown in Tables 1 and 2. The coefficients of variation based on the recovery of added inert aldosterone and cortisol were 23 and 12 % respectively (Table 1). The recovery values were obtained by subtracting the levels obtained before from those found after the addition of inert steroids to the urine, thus including the errors of both estimations. The coefficients of variation are therefore greater than would be given by repeated measurements on the same sample. Thus the estimated coefficients of variation of the individual aldosterone and cortisol values obtained in the recovery experiments are 15 and 8 % respectively, which are in good agreement with the mean coefficients of variation, 16 and 7 %, given directly by the duplicate data presented in Table 2.

The total variation which these coefficients represent consists of a combination of errors of radioactive counting, errors of the fluorescence measurements on the final paper chromatogram and, with aldosterone, differences in the recovery of this steroid from the stage of urine extraction up to that of adding the radioactive aldosterone diacetate. With cortisol, this last variation is eliminated by applying the isotope-dilution method throughout the entire procedure. As estimated from the variation in the recovery of radioactive cortisol (Table 1; i.e. 10 %, including radioactive counting errors) the error due to differences in recovery would be about 7 %. This could also be eliminated in the measurement of aldosterone if the radioactive free steroid were available for addition to the urine. In the present method, by adding radioactive aldosterone diacetate, it is only necessary to analyse the effluent fraction containing the peak concentration of this steroid in the second column.

The values for the 24 hr. excretion of aldosterone in hospital patients without endocrine disturbances, and in normal students, are higher by this method than those obtained by other workers employing different procedures. Since the lowest individual value in nineteen cases investigated by this method is higher than the mean values given by other workers, the difference is probably statistically significant. This could be the result of one or more of the following factors:

(*a*) The accidental selection of subjects in our series with abnormally high aldosterone values arising from dietary conditions or concealed endocrine disease. However, there is no evidence for this and the electrolyte balance of the subjects appeared to be normal.

(*b*) Decreased destruction of aldosterone in the urine at pH 1 owing to the use of the continuous extraction procedure.

(*c*) Increased recovery of aldosterone in the present method, possibly as a result of more efficient extraction or the use of partition-column chromatography as a separative method, rather than paper chromatography as employed by most other workers.

(*d*) The possible inhibition of the activity of aldosterone by interfering substances in those methods depending on bioassay.

At the present time there is not enough evidence to allow any firm conclusions to be drawn as to the relative importance of the above factors. However, Dr J. J. Walraven of Organon, Oss, Holland (private communication), has recently found that when continuous extraction is used as the first stage of the Neher–Wettstein method (Neher & Wettstein, 1955), the amounts of aldosterone obtained are 2–3 times those with their normal procedure on the same urine.

SUMMARY

1. A method is described for the analysis of aldosterone, cortisol and corticosterone after continuous extraction of human urine at pH 1 by chloroform for 24 hr. at room temperature.

2. An isotope-dilution method is used. Paper chromatography of the steroids or their acetyl derivatives follows their extensive purification by column chromatography. The final spot on the paper chromatogram is measured quantitatively by a fluorimeter after development with a solution of sodium hydroxide.

3. Evidence is presented on the specificity of the method.

4. Recovery of steroids throughout the procedure is of the order of 70 %.

5. The recovery and duplicate data suggest that the coefficient of variation for the determination of aldosterone and cortisol is 15 and 7 % respectively.

6. The mean values for the 24 hr. urinary excretion of aldosterone and cortisol have been found to be 11 μg. (31 cases) and 35 μg. (24 cases) respectively.

7. Some values found in pathological conditions are given.

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ADDENDUM

A Fluorescence Method for the Microanalysis of Δ^4-3-Oxo Steroids on Paper Chromatograms

By P. J. AYRES, SYLVIA A. SIMPSON and J. F. TAIT
Department of Physics Applied to Medicine and the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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The method for the analysis of aldosterone and cortisol, described in the preceding paper, depends finally on the assay of their acetyl derivatives after paper chromatography. The fluorescence of the areas containing steroid is measured without elution, after developing the chromatograms by the method of Bush (1952). The method is applicable to the micro-analysis of other Δ^4-3-oxo steroids, although the intensity of their fluorescence may be weaker, particularly for the more non-polar free compounds such as 11β-hydroxypregn-4-ene-3:20-dione and 17:-hydroxypregn-4-ene-3:20-dione. The apparatus and method have been demonstrated (Ayres, Simpson & Tait, 1956).

EXPERIMENTAL

Preparation of paper specimens

The chromatograms are sprayed on both sides of the paper with a 0·004% (w/v) solution of 3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride in 10% (w/v) NaOH. The chromatograms are then heated for about 15 min. at a distance of 50 cm. from a 2 kw electric fire with elements distributed evenly over an area 25 cm. square. When the chromatogram is still slightly damp, it is finally dried with hot air from a hair drier. It is essential that the preliminary heating be carried out in still air.

By viewing the chromatogram under ultraviolet light, the head of the template positioner (*g*, Fig. 1) is placed on either side of the paper with the crosswires arranged centrally over

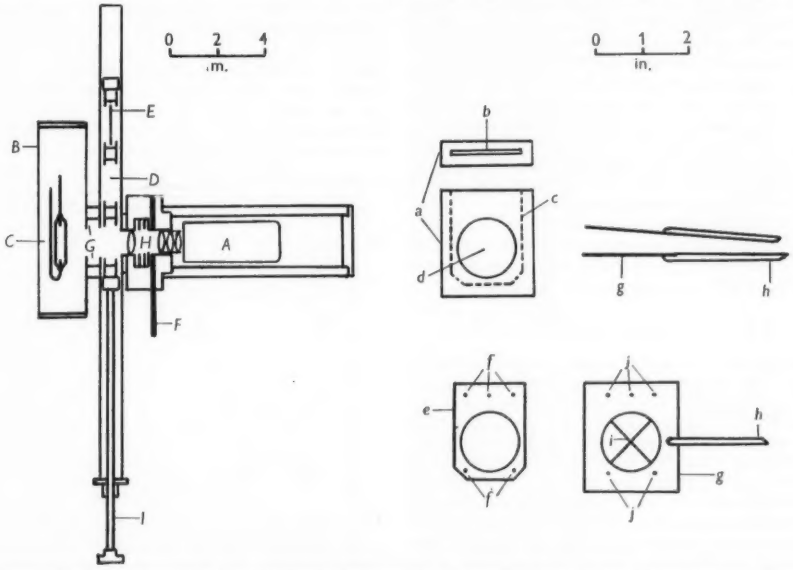

Fig. 1. Fluorimeter and accessories for measuring fluorescence of compounds on paper chromatograms. The diagram on the left shows the fluorimeter schematically: *A*, photomultiplier; *B*, lamp-housing; *C*, mercury-vapour lamp; *D*, compartment for paper holder (*a*); *E*, solid fluorescent standard; *F*, shutter; *G*, primary filter compartment; *H*, secondary filter compartment; *I*, sliding control for paper holders. The diagrams on the right show the accessories for positioning the fluorescent compound centrally: *a*, paper holder; *b*, slot for paper; *c*, outline of paper; *d*, aperture with central fluorescent spot; *e*, cutting template for paper chromatogram; *f*, positioning pins; *g*, template positioner; *h*, tongs; *i*, crosswire; *j*, positioning holes.

a fluorescent spot containing steroid. The handles of the positioner are then locked and a pin is pushed through the positioning holes to puncture the paper. A cutting template with a set of positioning pins is placed over the fluorescent spot with the pins fitting into the holes in the paper. The paper is now cut round the template to the shape shown at *e*, Fig. 1. The other spots are dealt with in the same way and in addition, for routine analysis, a suitable area not containing steroid is taken to serve as a blank measurement. The pieces of paper are then placed in paper holders (*a*, Fig. 1), each of which consists of a slotted square of brass with a circular aperture, 3·2 cm. in diameter. The fluorescent spots should be in the centre of this aperture. The paper holders can now be placed in the compartments (*D*, Fig. 1) of the fluorimeter.

Description of fluorimeter

The fluorimeter has a mercury-vapour lamp, an 80 w Mazda type MB/V, with glass envelope removed, as a light source. The light from this source (after passing through four Ilford 828 filters, total thickness 1·2 cm.) irradiates the paper or an Ilford 805 filter which serves as a solid fluorescent standard (*E*, Fig. 1). A quartz lens of focal length 2·5 cm. is placed 2·5 cm. from the fluorescent spot on the paper, so that the emitted light is made nearly parallel before entering the filter compartment. One Hilger and Watts interference filter (maximum transmission 5975Å), together with Ilford filters 401 and 607, is used as secondary filter. The light is then focused on the cathode (0·95 cm. in diameter) of an E.M.I. photomultiplier Type 6094 by three lenses of focal length 2·5, 3·2 and 3·2 cm. The optical arrangement ensures that fluorescent light emitted from any point in the circular aperture of the paper holder is focused on the photomultiplier cathode. The high-tension supply for the cathode and dynodes of the photomultiplier (usually 1700 v, negative with respect to earth) is supplied by an Atkins Robertson and Whiteford Type 1082 A power pack. A Pye Scalamp 7904/S galvanometer measures the current passing between the collector plate and ground potential. With this electrical arrangement it was found essential, if instability were to be avoided, to have a sheath maintained at cathode potential surrounding the glass body of the photomultiplier. The a.c. mains supply was stabilized by an Advance 250 w constant-voltage transformer.

Method for routine fluorimeter measurements

For routine analyses, four fluorescent spots are usually measured. These consist of two standards (0·5 and 1 μg. of aldosterone diacetate or 0·75 and 1·5 μg. of cortisol monoacetate) and two portions, in the ratio 2:1, of the steroid to be analysed. The high-tension supply to the photomultiplier is adjusted so that with the solid fluorescent source in position, the galvanometer gives a certain fixed reading (usually 60 mm. on the most sensitive range). This is repeated after every measurement of the fluorescent spots, to eliminate an error of about 2% due to variation of light-source intensity and photomultiplier sensitivity. The pieces of paper are now inserted in turn and the corresponding galvanometer readings noted. The sliding control (*I*, Fig. 1) moves the three paper holders laterally into position for this purpose, and three positioning notches ensure that the circular apertures are concentric with the photomultiplier cathode. If necessary, the paper holders can be moved laterally until a maximum reading of the galvanometer is observed. However, by using the method described for cutting the paper, this reading is invariably as defined by the positioning notches. The reading for the blank is now subtracted from those of the fluorescent spots. This results in a higher coefficient of variation for the measurement of the smaller amounts of steroids; the readings, corrected for blank, are therefore weighted in the final calculation by taking: [unknown amount of steroid (larger portion)]/[known amount of standard steroid (larger standard)] as (sum of two galvanometer readings of unknown amount of steroid)/(sum of two galvanometer readings for two standard spots).

Fluorescence spectra

Before the secondary filters for the fluorimeter were chosen, the spectral emissions of the soda-fluorescent spots of aldosterone diacetate and cortisol monoacetate, together with that of a blank piece of paper developed in the same way, were obtained. The pieces of paper were placed in front of the entrance slit of a Hilger Uvispek apparatus, which served as a monochromator, and irradiated with ultraviolet light of wavelength 365 mμ. obtained from the source and primary filters of the fluorimeter. The E.M.I. 6094 photomultiplier tube, employed in the fluorimeter, was used as the detector instead of the photocell of the Uvispek apparatus. Galvanometer readings were taken for emergent light of different wavelengths between 400 and 600 mμ. Both slit widths were maintained at 0·06 cm.

Fig. 2 shows the spectral emission of the soda fluorescence of a piece of blank paper and also that of 5 μg. of aldosterone diacetate corrected for this blank fluorescence. The filled-in points show the latter spectrum also corrected for the spectral sensitivity of the photomultiplier cathode by employing the data provided by the manufacturers. These are the major corrections necessary to obtain the true fluorescence spectrum as the dispersion is nearly constant throughout the wavelength range investigated.

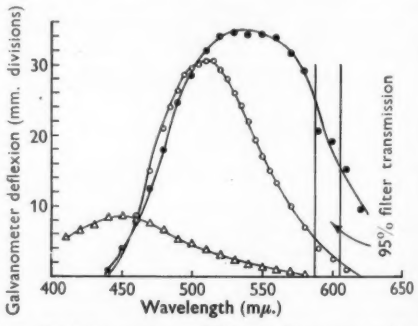

Fig. 2. Emission spectrum of the soda fluorescence of 5 μg. of aldosterone diacetate on a paper chromatogram. ○, The spectrum as measured by the photomultiplier used in the fluorimeter (cf. Fig. 1), corrected by subtracting the fluorescence of a corresponding area of paper not containing steroid; ●, the same spectrum corrected for both paper blank and spectral sensitivity of the photomultiplier cathode; △, the corresponding spectrum from paper not containing steroid as measured by the photomultiplier.

It can be seen that, as measured directly by the photomultiplier, the maximum fluorescence of the steroid occurs at 515 mμ., the corrected maximum being at 540 mμ. Both the corrected and measured maxima for the blank paper were at 450 mμ. Similar curves were obtained for cortisol monacetate. The longer the wavelength, the greater is the ratio of the intensity of fluorescence of steroid compared with that of the blank paper. However, because the sensitivity of the photomultiplier falls off rapidly above 590 mμ., filters were chosen which had 95% of their transmitted light between 585 and 605 mμ., as shown in Fig. 2.

RESULTS

Proportionality of galvanometer reading to quantity of steroid

Five chromatograms, each with four spots of 1, 2, 4 and 8 μg. of aldosterone diacetate, were developed and measured as previously described. In addition, the readings from twenty chromatograms with 0·5 and 1 μg. of aldosterone diacetate were available for analysis. The results are shown in Fig. 3. The values on the ordinate represent the mean values of several galvanometer readings corresponding to a particular quantity of steroid expressed as the ratio of that given by 1 μg. of steroid on the same chromatogram. This procedure was adopted because of the large variation of the galvanometer readings given by different chromatograms with a constant quantity of steroid. This variation is probably due to differences in the conditions of spraying and heating of different chromatograms, although the treatment of the total area of a single chromatogram may be uniform. Examination of the means and standard errors of the experimental points shown in Fig. 3 indicates that the galvanometer readings are proportional to quantity of aldosterone diacetate up to at least 4 μg., but that this may not be so for the readings corresponding to 8 μg. of steroid or more. More rigorous statistical treatment of the data confirms this conclusion.

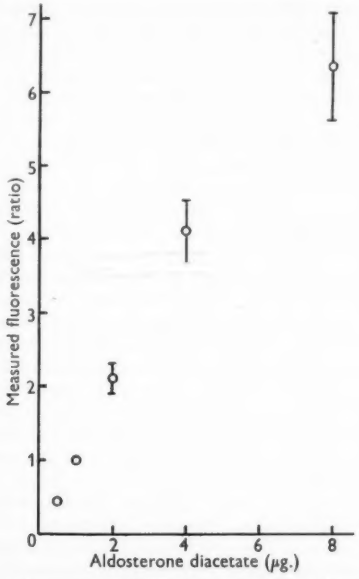

Fig. 3. Fluorescence of different quantities of aldosterone diacetate. Values on the ordinate represent the mean values of several measurements of fluorescence expressed as the ratio of that given by 1 μg. of steroid on the same chromatogram. The length of the vertical lines attached to the points represents twice the standard error of the mean. The point corresponding to 0·5 μg., i.e. $0{\cdot}478 \pm 0{\cdot}01_3$ (S.E.), represents the mean of the results from twenty chromatograms; other points (2, 4 and 8 μg.) are the mean results from five chromatograms.

Effect of impurities

Cortisol was extracted and measured from 1 l. of urine exactly as described in the preceding paper, except that the urine was first adjusted to pH 6. The same urine was then further continuously extracted at pH 1. About 10% of the 2 μg. of [4-^{14}C]cortisol added initially to the urine was extracted in the second process, presumably owing to incomplete preliminary recovery. This enabled a second measurement of the cortisol to be made. However, whereas the first paper chromatogram from the extraction at pH 6 showed no fluorescent- and very few ultraviolet-absorbing impurities, the second paper chromatogram from the extraction at pH 1 showed some running even at the same speed as the cortisol monoacetate spot, although these did not give yellow fluorescence. In the analysis of six urines carried out in this manner, the mean ratio of the values given by extraction at pH 1 and 6 was $1{\cdot}03_5 \pm 0{\cdot}05$ (S.E.).

Sensitivity

Twenty chromatograms, with 0·5 μg. spots of aldosterone diacetate, gave galvanometer readings of $10{\cdot}1 \pm 1{\cdot}1$ (S.D.) after correction for blank fluorescence. Two chromatograms, with four spots of 0·5 μg. on one and four of 0·25 μg. of aldosterone diacetate on the other, were measured. Four blank areas were also measured for each chromatogram. The mean and standard deviations for the readings were 0·5 μg. of steroid, $14{\cdot}1 \pm 1{\cdot}0$, corresponding paper blanks, $7{\cdot}4 \pm 1{\cdot}0$; and 0·25 μg. of steroid, $10{\cdot}1 \pm 1{\cdot}2$, corresponding paper blanks, $6{\cdot}8 \pm 1{\cdot}1$.

Precision

Two batches of aldosterone diacetate were compared in a typical series of determinations. One batch was prepared from a specimen of aldosterone,

kindly supplied by Professor Reichstein, Basle. The other was prepared by the authors from Allen and Hanbury's adrenal extract. Both preparations of aldosterone diacetate were purified by partition-column chromatography and standardized by ultraviolet absorption. Nine chromatograms were used for the comparison with 0·5 and 1 μg. of both specimens on every sheet. They were developed, measured and the results calculated as previously described for routine analyses. The mean ratio of London/Basle aldosterone, as found by this method, was 0·98, with a standard deviation of 0·062 for the nine determinations. The mean coefficient of variation for determinations carried out in this manner with pure steroids was therefore about 6 %.

Effect of spot size

Two chromatograms, with four spots of 2 μg. of aldosterone diacetate on both sheets, were run, developed and measured. The final diameter of the spot was varied by altering the area of application of the steroid solution at the starting line of the chromatogram. The final spot diameter and corresponding galvanometer readings (in parentheses), corrected for blank fluorescence, were: 0·6 cm. (20 mm.); 1·0 cm. (21 mm.); 1·4 cm. (20 mm.) and 1·8 cm. (19 mm.) for one chromatogram, which included the range of spot sizes normally encountered in analysis, and 1·0 cm. (14 mm.); 2·0 cm. (12 mm.); 3·0 cm. (8 mm.) and 4·0 cm. (6 mm.) for the other chromatogram.

These results again clearly show the variation of galvanometer reading between chromatograms for the same quantity of steroid.

DISCUSSION

The comparison of the two different specimens of pure aldosterone diacetate gives a coefficient of variation of 6 % for the fluorimetric method. The coefficients of variation in the determination of urinary cortisol and aldosterone by the complete method, described in the preceding paper, are 7 and 15 % respectively. The random errors involved in the analysis of cortisol in urine are probably a combination of those involved in radioactive and fluorescence measurements. As the radioactive counting errors are about 4 % (total counts of approximately 600), the coefficient of variation for the fluorimetric measurement of cortisol monoacetate from urine can be estimated as 6 %. The random errors in the analysis of aldosterone are probably a combination of these plus the variation in recovery, which is not fully corrected for by the isotope-dilution method in the determination of this steroid. An indication of the variation in recovery of aldosterone is given by the coefficient of variation in the recovery of radioactive cortisol, i.e. 10 %. If this figure is taken for the recovery and 4 % for the radioactive errors, the coefficient of variation of the fluorimetric method can be estimated as about 10 %. Thus the coefficient of variation of the method, as applied to the determination of pure aldosterone diacetate or crude cortisol monoacetate and aldosterone diacetate prepared from urine by column chromatography, would appear to be between 6 and 10 %.

The major reasons for likely systematic errors in the method are the possible quenching action of impurities, the effect of spot size and other effects leading to lack of proportionality of galvanometer reading to quantity of steroid.

For the measurement of pure steroids, the results previously described make it likely that these errors will not occur if the spot obtained after developing the chromatogram contains less than 4 μg. of steroid and is less than 2 cm. in diameter. For the measurement of impure fractions, not only must these conditions be satisfied but, in addition, impurities must not enhance or quench the fluorescence. The correlation of the concomitant bioassays and physicochemical results suggests that these criteria are being followed in the application of the method to the analysis of aldosterone in human urine. The agreement between results for the recoveries of inert and radioactive cortisol added initially to the urine indicates that this is also true for measurements of this steroid. However, in both these cases the fluorimetric method has been applied only after extensive purification, and it may be dangerous to assume that systematic errors do not occur for other applications of the method. However, even when many impurities are present in the final chromatogram such as after extraction of free cortisol from urine at pH 1 following extraction at pH 6 as described in the Results section, no interference can be detected. This conclusion is based on the agreement between the measured specific activity of the cortisol after extraction at either pH 1 or 6 and assumes that cortisol is not significantly increased by acid hydrolysis, an assumption which accords with the results of other workers using different methods.

The measurement of Δ^4-3-oxo steroids on paper chromatograms by the soda-fluorescence method has previously been carried out by visual examination of the resultant spots. The present method has the advantage that an objective measurement is made. However, the specificity of the reaction as discussed elsewhere (Bush, 1954) is not affected by this modification.

A reaction which appears to be similar in principle and specificity and which can be carried out in solution has recently been described (Abelson & Bondy, 1955). This involves the development of yellow fluorescence by the action of a solution of

potassium *tert.*-butoxide in *tert.*-butanol on a Δ^4-3-oxo steroid. This solution method may be more convenient for measuring relatively pure steroids. The paper method, however, has the advantage that additional separation of steroids is obtained during the analytical procedure and the presence of compounds, other than the one to be measured, is revealed; this method may therefore be preferable for the analysis of impure fractions.

It seems likely that the method for measuring fluorescent compounds on paper can be applied to substances other than Δ^4-3-oxo steroids. During the preparation of this paper our attention was drawn to the description of such an application by other workers (Mourodineanu, Sanford & Hitchcock, 1955).

SUMMARY

1. A method has been described in which amounts of Δ^4-3-oxo steroids greater than 0·2 μg. can be estimated by measuring their fluorescence after chromatography on paper and development by a solution of sodium hydroxide.

2. The intensity of fluorescence is proportional to the quantity of steroid if the final spot contains less than 4 μg. of steroid and is less than 2 cm. in diameter.

3. If four spots (two of them standards) are used for an analysis, the coefficient of variation of the estimate is between 6 and 10 %.

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The Reactions of Diazonium Compounds with Amino Acids and Proteins

By A. N. HOWARD and F. WILD
Department of Medicine, University of Cambridge

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The reaction between diazonium compounds and proteins in alkaline solution was first studied in 1904, with sulphanilic acid as the amino compound (Pauly, 1904). Coloured compounds were formed, and the reaction was investigated by allowing the diazonium compound to react separately with all the amino acids then known to be present in proteins. It was found that only tyrosine and histidine gave coloured solutions. Later, Pauly (1915) isolated derivatives from these two amino acids with diazotized *p*-arsanilic acid.

In all azoproteins prepared by treating proteins with an excess of diazotized *p*-arsanilic acid, more arsenic was found on analysis than is to be expected from the combined tyrosine and histidine content (Hooker & Boyd, 1934; Boyd & Mover, 1935; Kapeller-Adler & Boxer, 1936). In an attempt to explain the results it was suggested that diazonium compounds could also react with phenylalanine, tryptophan, proline and hydroxyproline. Kapeller-Adler & Boxer (1936) reported that they had prepared and isolated derivatives from these amino acids with diazotized *p*-arsanilic acid.

The reaction of diazotized *p*-sulphanilic acid with horse serum and compounds with groupings found in proteins has been investigated (Eagle & Vickers, 1936). Indole, the aliphatic amino group of glycine, α-alanine, phenylalanine, ornithine and lysine and the imino group of proline and hydroxyproline were found to deactivate the diazonium compound, from which it was concluded that reaction occurs with aliphatic amino and imino groups in proteins but not with peptide linkages, guanidino or amide groupings. No attempt was made to prepare derivatives of compounds containing the groupings mentioned above: the destruction of the diazonium compound was followed by the addition of alkaline β-naphthol.

Derivatives of glycine have been isolated by treating it with diazotized aniline and nitroaniline (Busch, Patrascanu & Weber, 1934). Subsequently derivatives have been isolated from the products of

interaction of diazotized 5-chloro-2-methylaniline and (*a*) proline, (*b*) cysteine respectively (Zahn, Wollemann & Waschka, 1954). Zahn *et al.* also state that with aliphatic amino acids a diazoaminobenzene is formed.

In the present paper the reaction of diazonium compounds with amino acids and compounds containing groupings present in proteins is described. Derivatives have been prepared and isolated, and the influence of the position of the amino group on the nature of the product of reaction has been studied. The results of these experiments have been compared with analytical data on the products obtained by coupling proteins with diazotized *p*-arsanilic acid.

RESULTS

Reaction with amino groups

Glycine was found to react with diazotized aniline in alkaline solution to form bisbenzenediazoglycine, $(C_6H_5.N{:}N)_2N.CH_2.CO_2H$. A small amount of diazoaminobenzene also was produced. It might be expected that α-amino acids other than glycine would react similarly, but our experiments have shown that this is not so. Three α-amino acids, namely α-alanine, phenylalanine and leucine, gave diazoaminobenzene as the main product and no triazen was formed. The conversion of a diazonium compound into a diazoamino compound by α-amino acids could be explained by the following series of reactions:

$$C_6H_5.N_2.OH + R.CH(NH_2).CO_2H \rightarrow C_6H_5.NH_2 + R.CH(OH).CO_2H + N_2;$$

$$C_6H_5.NH_2 + C_6H_5.N_2.OH \rightarrow C_6H_5.N_2.NH.C_6H_5 + H_2O.$$

Support for this mechanism is provided by the isolation of DL-phenyllactic acid in the experiments with DL-phenylalanine. The hydroxy acid was prepared from the amino acid and pure crystalline diazobenzene hydrogen sulphate to prevent its formation from excess of nitrous acid used in diazotization. Further, the percentage yield of DL-phenyllactic acid was comparable with that of diazoaminobenzene. No evidence was obtained that the diazoaminobenzene and α-hydroxy acid are formed by the spontaneous decomposition of the aliphatic triazen, for bis(benzenediazo)glycine was quite stable in cold sodium carbonate solution, as were the other aliphatic triazens which were prepared.

An investigation was made of the influence of the position of the amino group in the acid chain on the nature of the products. In experiments with very dilute solutions (about 1%) of methylamine and ethylamine in M sodium carbonate solution, the bis(benzenediazoamino)amine was formed almost quantitatively. Amino acids in which the amine grouping is terminal but not in the α-position, such as β-alanine, γ-aminobutyric acid, ε-aminocaproic acid and glycylglycine, all gave high yields of the bis(benzenediazoamino) compound, with no detectable formation of diazoaminobenzene. It is concluded, therefore, that compounds containing the $-CH_2.NH_2$ grouping all react to form aliphatic triazens.

An amino acid in which the amino group was neither terminal nor in the α-position, such as β-aminobutyric acid, gave diazoaminobenzene as the chief product, and in contrast to the above-mentioned amino acids gave no evidence of triazen formation. A similar result was obtained with *iso*-propylamine, and it is concluded that compounds containing the group $CH_3.CH(NH_2).CH_2-$ do not form bis(benzenediazo)amino compounds.

It was noted that the rate of formation of diazoaminobenzene was slow, several hours being necessary for complete reaction. It is suggested, therefore, that diazoaminobenzene is produced only when the amino compound does not form a triazen or forms one slowly as does glycine. The groupings adjacent to the amino group affect the rate of reaction with diazonium salts, and the following combinations cause a decrease in reactivity:

CH_3-, $-CO_2H$; $C_6H_5.CH_2-$, $-CO_2H$;
$(CH_3)_2CH.CH_2-$, $-CO_2H$; CH_3-, $-CH_3$;
CH_3-, $-CH_2.CO_2H$.

Our experiments with diazotized *p*-arsanilic acid have not been as extensive or as detailed as those described with aniline. The diazonium compound is difficult to work with and the products of reaction are insoluble in non-polar and unstable in polar solvents. Two derivatives, however, were prepared as acids from methylamine and *n*-butylamine, and three more from amino acids were isolated as their sodium salts. A complete analysis was not possible on the last three compounds, owing to the co-precipitation of small amounts of inorganic salts, but the As/N quotients indicated formation of products analogous to those obtained with diazotized aniline: further, DL-phenyllactic acid was isolated from the reaction with DL-phenylalanine in good yield.

Reaction with guanidino groupings

Three equivalents of diazotized aniline were added to methylguanidine, and benzenediazomethylguanidine was isolated. The structure of this compound is probably represented by

$$C_6H_5.N_2.NH.C(:NH).NH.CH_3,$$

analogous to the derivative prepared from dicyanodiamide by Walther & Grieshammer (1915).

Although a large excess of the phenyldiazonium salt was used there was no evidence that further reaction could take place.

Reaction with sulphydryl and aliphatic hydroxyl groups

In an experiment with *p*-diphenylylamine and ethanethiol, a compound was isolated which contained no nitrogen, but had a sulphur content corresponding to that of ethyl *p*-diphenylyl thio ether. This compound was presumably formed from the extremely unstable *S*-azo derivative.

Reaction with indolyl groups

Experiments with 3-indolylacetic acid showed that reaction with indoles substituted in the 3-position takes place to give an unstable product. It seems probable that the diazonium salt reacts with 3-indolylacetic acid at the imino group to form a triazen, and that the triazen can undergo rearrangement to form a coloured *C*-azo compound.

Reaction with proteins

Effect of pH *on the reaction.* Solutions obtained by diazotizing 0·25 g. or 0·5 g. of *p*-arsanilic acid were added to 0·25 g. of casein dissolved in 12·5 ml. or 25 ml. respectively of the following reagents: M-$NaHCO_3$, 0·5M-Na_2CO_3, M-Na_2CO_3, M-Na_2CO_3 and N-NaOH (1:1, v/v). The pH was determined with a glass electrode and the products were treated as described in the Experimental section. The results (Table 1) show that a product with the highest As/N quotient was obtained when the reaction was carried out at pH 9·8.

Maximum reaction at pH 9·8. *p*-Arsanilic acid (0·25 g.) was diazotized and added to a cold soln. of 0·25 g. of protein in 12·5 ml. of M-Na_2CO_3. Multiples of this amount of *p*-arsanilic acid were used with the same weight of protein dissolved in correspondingly larger volumes of M-Na_2CO_3. A series of compounds was thus prepared for each of four proteins, with the quotient *p*-arsanilic acid/protein (w/w) ranging from 1 to 6. The analytical results are shown in Table 2. The As/N quotients in the products

Table 1. *Variation of arsenic/nitrogen quotient of casein conjugated with* p*-arsanilic acid at various* pH *values*

Alkaline solution	pH	*p*-Arsanilic acid (g./g. of protein)	Analysis of product: As (%)	N (%)	As/N
M-$NaHCO_3$	7·5	1	7·1	11·6	0·612
0·5M-Na_2CO_3	9·3	1	8·9	11·1	0·802
0·5M-Na_2CO_3	9·1	2	9·5	11·6	0·819
M-Na_2CO_3	9·8	1	10·2	12·3	0·848
M-Na_2CO_3	9·8	2	11·1	11·7	0·949
M-Na_2CO_3–N-NaOH	11·5	1	4·6	11·8	0·390

Table 2. *Arsenic/nitrogen quotients of proteins conjugated with diazotized* p*-arsanilic acid at* pH 9·8, *and comparison with values theoretically possible*

Protein	*p*-Arsanilic acid (g./g. of protein)	Analysis of product: As (%)	N (%)	As/N	Theoretical values for As/N*: 1	2	3
Casein	4	11·5	11·7	0·983	0·939	0·837	0·440
	3	11·7	11·9	0·983			
	2	11·1	11·7	0·949			
	1	10·2	12·3	0·848			
Ovalbumin	4	10·8	11·8	0·915	0·883	0·721	0·300
	3	11·1	12·0	0·925			
	2	10·1	12·0	0·855			
Edestin	6	12·1	12·1	1·000	1·034	0·678	0·289
	4	12·1	12·2	0·992			
	3	11·6	12·4	0·936			
	2	11·2	12·3	0·911			
Bovine-plasma albumin	4	12·7	11·6	1·095	1·088	0·968	0·424
	3	12·7	11·6	1·095			
	2	11·8	12·1	0·975			
	1	9·6	12·4	0·774			

* Column 1. Theoretical values of As/N assuming that 2 moles of diazotized *p*-arsanilic acid react with 1 mole of phenolic, imidazole, α-amino groups of terminal glycine residues, ε-amino groups; 3 moles with 1 mole of guanidino group and 1 mole with indolyl and sulphydryl groups, also assuming deamination of α-amino groups of terminal amino acids other than glycine. Column 2. As above, except 1 mole of diazotized *p*-arsanilic acid assumed to react with 1 guanidino group. Column 3. Assuming 2 moles of diazotized *p*-arsanilic react with 1 mole of phenolic and imidazole groups only.

obtained are compared with those theoretically possible from the amino acid compositions of the proteins given by Tristram (1953).

Stability of the azoproteins. The ppt. (0·5 g.) from casein coupled with 1 g. of diazotized *p*-arsanilic acid/g. of protein and having a value for the As/N quotient of 0·848 was dissolved in 25 ml. of 0·5 M-$NaHCO_3$. This solution was divided into two parts; one part, left at 5° for 24 hr. and then treated in the normal manner, gave a product with an unchanged As/N quotient of 0·842, but the other, kept at 37° for the same time, gave a product with a diminished As/N quotient of 0·765. A product obtained from bovine-plasma albumin, having an initial As/N quotient of 0·774, was also dissolved in $NaHCO_3$. After 24 hr. at 5° the value was also unchanged, but after the same time at 56° it had changed to 0·536. These azoproteins therefore contain linkages unstable to heat.

Homogeneity of the azoproteins. The azoproteins (10 mg.), obtained from diazotized *p*-arsanilic acid and casein, ovalbumin, edestin, and bovine-plasma albumin, were dissolved in 0·5 M-$NaHCO_3$ (about 5 ml.). A sample (0·01 ml.) of each solution was applied to Whatman no. 1 paper, and electrophoresis was carried out in a veronal buffer (pH 8·6, $I = 0·05$) for 7 hr. with a current of 2 mA/5 cm. strip and potential gradient of 5 v/cm. Typical products from each of the purified azoproteins were thus developed and in none was more than one band, orange-coloured, observed. Migration occurred towards the anode compartment and for proteins coupled with 4 g. of *p*-arsanilic acid/g. of protein the distances moved were as follows: casein, 5·5 cm.; ovalbumin, 6·1 cm.; bovine-plasma albumin, 4·9 cm.; edestin 5·8 cm.

DISCUSSION

Diazonium compounds have been used extensively to produce artificial antigens, Landsteiner & Lampl (1917, 1918) being the pioneers in this field. A more recent use of the method is demonstrated in papers in which the present authors collaborated (Coombs, Mynors & Wild, 1951; Britton, Coombs, Mynors & Wild, 1951; Coombs, Howard & Wild, 1952; Coombs, Howard & Mynors, 1953). The reactions of diazonium compounds with histidine and tyrosine were the first reactions to be investigated and derivatives were isolated by Pauly (1915) from the highly coloured reaction mixtures. It has not been realized previously that coupling reactions with other amino acids are possible to give colourless products, and a recent review (Herriott, 1947) has emphasized the incomplete state of present knowledge of this reaction. It was thought that further work was necessary in the investigation of the reactions of diazonium compounds with individual amino acids and the correlation of these results with the possible reactions with proteins. The results published in the early literature are considered in this paper and explained in a general reaction mechanism.

The isolation of an aliphatic triazen by the reaction of an amino acid with a diazonium compound was first reported by Busch *et al.* (1934), who isolated bis(benzenediazo)glycine from the reaction of diazotized aniline and glycine in neutral solution. They reported in addition that no product was formed when excess of sodium hydroxide was present. We have confirmed these results but have noted that in weak alkali, such as sodium carbonate, the yield of triazen is similar to that obtained in neutral solution. Like Busch *et al.* (1934), we have also isolated diazoaminobenzene from the products of the reaction.

Glycylglycine was found to react under the same conditions as glycine to form a triazen exclusively, no diazoaminobenzene being formed. As the reaction for proteins is usually carried out in weak alkali, these results indicate that proteins containing *N*-terminal glycine residues could react to form bis(diazo)amino linkages.

Not all amino acids were found to behave like glycine in their reaction with diazonium compounds. In experiments with diazotized aniline and diazotized *p*-arsanilic acid, only those compounds containing a $-CH_2.NH_2$ group were found to form triazens. Amino compounds containing the group $R.CH(NH_2)-$ gave a diazoaminobenzene as the chief product. The latter type of reaction is not peculiar to amino acids, since Bucherer & Wolf (1904) found that ammonia and ammonium acetate convert diazotized *p*-nitroaniline quantitatively into *pp'*-dinitrodiazoaminobenzene. These workers suggested that *p*-nitroaniline was formed in the reaction and reacted spontaneously with unchanged diazonium compound. The application of this hypothesis to the reaction of certain amino acids with diazonium compounds entails the conversion of the amino acid into a hydroxy acid. Our experiments support the view that this change occurs, since we isolated phenyllactic acid from the products of the reaction with phenylalanine.

Zahn *et al.* (1954) have also found that valine, glycine and leucine give a diazoaminobenzene when allowed to react with diazonium compounds. Our results are in agreement with their findings, but not all amino acids give diazoaminobenzenes with diazonium compounds. Apart from glycine none of the compounds they used contained a $-CH_2.NH_2$ group, which we find gives a triazen and not (except with glycine) a diazoaminobenzene.

ϵ-Aminocaproic acid was among the compounds which were found to give a triazen. This result would indicate that in the diazo reaction with proteins reaction could occur to form bis(diazo)amino links with ϵ-NH_2 contributed by lysine.

α-Amino acids other than glycine have been found not to form triazens. In proteins which contain amino groups contributed by α-amino acids other than glycine, attack by the diazonium compound would be expected to proceed by the proposed 'deamination' mechanism. This would involve the deamination of the amino group.

Our results are in complete disagreement with those of Kapeller-Adler & Boxer (1936), who described the preparation of a derivative of phenylalanine in which two moles of diazotized *p*-arsanilic acid combined with one mole of the amino acid. The proposed structure of the highly coloured material was that of a *C*-azo compound, analogous to that of the tyrosine derivative previously isolated by Pauly (1915). We have found that the chief products of the above reaction are phenyllactic acid and sodium *pp'*-diazoaminobenzenearsonate.

Diazonium compounds can also react with the guanidino group. In experiments with methylguanidine and diazotized aniline, a new derivative, benzenediazomethylguanidine, was isolated. This confirms the early work of Walther & Grieshammer (1915), who showed that the guanidino group of dicyanodiamide, $H_2N.C(:NH).NH.CN$, could form a derivative with diazonium compounds. Similar compounds prepared from guanidinotaurine have been reported in the patent literature (Lecher & Orem, 1939). Although we have found that at least one diazonium group reacts with one guanidino group, it is our opinion that further reaction involving a total of three diazonium groups and one guanidino group could occur since the latter contains three active hydrogen atoms. Failure to obtained further reaction with methylguanidine was probably due to the extreme insolubility of the product, benzenediazomethylguanidine, in the aqueous media.

The isolation of a new derivative from diazotized *p*-diphenylamine and ethanethiol confirms the work of Stadler (1884), who showed that sulphydryl groups give unstable *S*-azo derivatives with diazotized *p*-sulphanilic acid and diazotized aniline. Similar *S*-azo compounds have been obtained by Friedländer & Chwala (1907). Sulphydryl groups in proteins would be expected to react in a similar manner.

The reaction of aliphatic hydroxyl groupings was not examined, as it has been fully investigated already (Hantzsch, 1898). It is possible that hydroxyl groups in proteins may be oxidized slowly to aldehydic groupings by diazonium compounds when present in excess.

Aliphatic imino compounds have been shown by Jacobs & Heidelberger (1921) to form diazoimino linkages with diazotized *p*-arsanilic acid. Furthermore, derivatives of proline with diazonium compounds involving the same linkage has been reported in the patent literature (Markush, 1933). In proteins, however, the free imino groups of proline and hydroxyproline occur only if these amino acids are present as end groups. The results of these workers do not support the claim of Kapeller-Adler & Boxer (1936) that highly coloured *C*-azo compounds can be formed from proline and hydroxyproline.

It has been suggested by Pauly & Gundermann (1908) that tryptophan and other 3-substituted indoles do not react with alkaline solutions of diazonium compounds, as the colour which is produced by tyrosine and histidine is not given by tryptophan. Our experiments with 3-indolylacetic acid have shown that reaction with 3-substituted indoles such as tryptophan can occur. Although the product was too unstable in acid to isolate, it is suggested that the tryptophyl group in proteins would react with at least one diazonium group.

All other groupings known to be present in proteins, including the amide linkage, have been considered and no evidence that they react with diazonium compounds has been obtained. We believe that the only reactions possible are those considered above.

The capacity of proteins to couple with diazotized *p*-arsanilic acid has been closely investigated by several workers (Hooker & Boyd, 1934, Boyd & Mover, 1935, Kapeller-Adler & Boxer, 1936), but we believe the results as yet reported are inaccurate and that the explanation of the reactions is incomplete. The extent of coupling has been calculated from the arsenic/nitrogen quotient of the azoprotein, but as an excess of the diazo compound is used to ensure complete reaction with the protein it is essential to carry out the analyses on a derivative which is not contaminated with decomposition products of diazotized arsanilic acid and to use methods of analysis which are accurate. The method of purification, therefore, is of great importance. Kapeller-Adler & Boxer (1936) precipitated the azoproteins by acidifying the aqueous alkaline reaction mixture. It is certain, therefore, that the derivatives used in their analyses were impure, for we have found that if *p*-arsanilic acid is diazotized, left to stand in alkaline solution for a number of hours and then acidified, a copious brown precipitate is formed. The precipitate will dissolve in alkali but is reprecipitated on acidification. Hooker & Boyd (1934) acidified an 80% alcoholic solution, in which the decomposition products of the diazotized *p*-arsanilic acid are soluble, and so prevented coprecipitation. The percentage of nitrogen in the azoprotein was determined by the Kjeldahl method, and it is accepted that this method of estimation gives a low value when the azo linkage is present (Kirk, 1950). We have repeated the preparations and estimations of the previous workers and confirm

that Kjeldahl values for nitrogen in the azoproteins containing a large number of azo linkages may be 20% lower than those found by the micro-Dumas estimation.

In our experiments diazotized *p*-arsanilic acid was coupled with casein, ovalbumin, edestin and bovine-plasma albumin, under the conditions described previously by Hooker & Boyd (1934). In addition, the solution of azoprotein was dialysed, which removed a further amount of impurity.

The precipitated azoproteins were not dried under vacuum, as the possibility existed of loss of nitrogen by this procedure. Analyses were done on the material dried in air, in contrast to the technique of analysis of the solution (Hooker & Boyd, 1934).

Kapeller-Adler & Boxer (1936) have reported that analysis for nitrogen by the micro-Dumas estimation gives a lower value than that obtained by Friedrich's method. We have not confirmed these findings. In our experiments only a small difference was noted and the micro-Dumas method gave the higher value. The estimation of the percentage of nitrogen, therefore, has been done by the micro-Dumas method.

Preliminary experiments were carried out to find the optimum pH for the reaction. It was found that the maximum coupling takes place at about pH 10. The main experiments were carried out at pH 9·8, so that it was possible to use the smallest amount of the diazotized *p*-arsanilic acid and obtain the greatest amount of coupling. We found that for complete reaction, 1 g. of casein, ovalbumin, or bovine-plasma albumin needed 3 g. of the diazonium compound, whereas edestin required 4 g. The derivatives so prepared were homogeneous and stable in the solid state. They could be stored in solution at 5°, but when kept at either 37° or 56° for some time the arsenic/nitrogen quotient fell considerably, showing that some of the linkages were labile to heat.

From the evidence considered above, it seemed probable that the reaction of proteins with diazonium compounds would occur through groupings other than the phenolic and imidazole groups which have been assumed to be involved since the work of Pauly (1904). The experimental values for the arsenic/nitrogen quotients were compared with the theoretical values on the assumption that reaction can take place with other groups present. On the basis of these experiments, we now propose that the phenolic group of tyrosine, the imidazole group of histidine and the ε-amino group of lysine react with 2 moles of a diazonium compound; that the sulphydryl group of cysteine and the indolyl group of tryptophan react with 1 mole; and that the guanidino group of arginine reacts with at least 1 and probably 3 moles. The α-amino group of glycine reacts with 2 moles of the diazonium salt when this amino acid is present as a terminal residue, but the α-amino grouping of other amino acids present are deaminated. Our experiments show that the $-CH_2.NH_2$ group must be present to allow the formation of a triazen. In the proteins we have used, the imino groupings of proline and hydroxyproline do not occur as end groups, but it is probable that if proteins containing such groupings were used reaction would take place between the *N*-terminal residue and 1 mole of the diazo reagent.

The experimental and theoretical arsenic/nitrogen quotients are in close agreement, for the proteins used, on the assumption that coupling takes place in this manner and that the guanidino group reacts with a maximum of 3 moles of the diazo reagent. In particular, this is true for edestin with its very high arginine content.

EXPERIMENTAL

Unless otherwise stated, 'light petroleum' refers to solvent boiling within the range 60–80°.

Reactions with diazotized aniline

An aqueous solution of benzenediazonium chloride was prepared by dissolving 1·2 g. of aniline hydrochloride in 25 ml. of water and 2 ml. of conc. HCl. To the ice-cold solution was added 11·3 ml. of M-$NaNO_2$ slowly and the mixture kept at 5° for 15 min. The diazo solution was added to the following compounds, in the quantities indicated, dissolved in 50 ml. of cold M-Na_2CO_3. The reaction mixtures were left overnight at 4°.

(*a*) *Primary amino compounds which react with diazotized aniline to form triazens.* (i) Methylamine (0·64 g. of methylamine hydrochloride). A yellow oil separated rapidly and on standing solidified to a red solid. Light-yellow crystals of bis(benzenediazo)methylamine were obtained after recrystallization from light petroleum, the solution being decolorized with charcoal; yield 90%; m.p. 113–114°. (Found: N, 29·2. Calc. for $C_{13}H_{13}N_5$: N, 29·3%.) Goldschmidt & Badl (1889) give m.p. 112–113°.

(ii) Ethylamine (0·77 g. of ethylamine hydrochloride). A yellow oil separated immediately and solidified. Light-yellow crystals of bis(benzenediazo)ethylamine were obtained after recrystallization from aqueous ethanol, the solution being decolorized with charcoal; yield, 90%; m.p. 69·5°. (Found: N, 27·6. Calc. for $C_{14}H_{15}N_5$: N, 27·7%.) Goldschmidt & Badl (1889) give m.p. 70–71°.

(iii) Glycine (0·345 g.). A red–orange solution with some brown solid was obtained. The filtered solution was acidified with 2·5 N-HCl and the yellow precipitate formed filtered, washed with water and dried. Light-yellow crystals of bis-(benzenediazo)glycine were obtained by crystallization from benzene; yield 35%; explodes 109°. (Found: N, 24·5. Calc. for $C_{14}H_{13}O_2N_5$: N, 24·7%.) Busch *et al.* (1934) give decomp. 124°. The brown solid from the reaction mixture was dried; light-yellow crystals of diazoaminobenzene were obtained after recrystallization from light petroleum and decolorizing with charcoal; yield 10%; m.p. 99° (unchanged by admixture with an authentic sample, m.p. 99°).

(iv) β-Alanine (0·5 g.). A yellow solid separated after 2 min. This solid was completely soluble in a large excess of

water and was presumably the sodium salt of the compound formed. The reaction mixture was acidified with 2·5 N-HCl and the resulting solid filtered, washed with water and dried, giving a light-brown powder. Light-yellow crystals of bis-(benzenediazo)-β-alanine were obtained after recrystallization from benzene–light petroleum; yield 65%; decomp. 101° (Found: N, 23·4. $C_{15}H_{15}O_2N_5$ requires N, 23·6%.)

The reaction of γ-aminobutyric acid, ε-aminocaproic acid and glycylglycine was similar to that described above for β-alanine. The treatment was in every respect identical except for the solvents used in recrystallization.

(v) γ-Aminobutyric acid (0·474 g.). Yellow crystals of bis(benzenediazo)-γ-aminobutyric acid were obtained after recrystallization from light petroleum; yield 60%; m.p. 103° (decomp.). (Found: N, 22·4. $C_{16}H_{17}O_2N_5$ requires N, 22·5%.)

(vi) ε-Aminocaproic acid (0·603 g.). Yellow crystals of bis(benzenediazo)-ε-aminocaproic acid were obtained after recrystallization from light petroleum, yield 60%; m.p. 93°. (Found: N, 20·5. $C_{18}H_{21}O_2N_5$ requires N, 20·7%.)

(vii) Glycylglycine (0·625 g.). Light-yellow crystals of bis(benzenediazo)glycylglycine were obtained after recrystallization from benzene–light petroleum; yield 70%; decomp. 105°. (Found: N, 24·6. $C_{16}H_{16}O_3N_6$ requires N, 24·7%.)

(*b*) *Amino compounds which yield diazoaminobenzene as the chief product.* (i) α-Alanine (0·42 g.). After the addition of the diazotized aniline, the reaction mixture became opalescent and within 10 min. a ppt. formed which gradually increased over several hours. A continuous evolution of N_2 occurred throughout the reaction. (A Na_2CO_3 soln. without α-alanine gave no visible evolution of N_2 gas or opalescence until after 1 hr.) The yellow–brown ppt. was filtered off and dried. The light-yellow filtrate gave no ppt. on acidifying with 2·5 N-HCl. Since recrystallization proved ineffective, the brown product was purified in the following way. Alumina slurry with light petroleum was used to prepare a column 30 cm. diam., 150 cm. in height. The product (300 mg.) was dissolved in 20 ml. of light petroleum and added to the column, which was developed with light petroleum. A yellow band separated from a stationary black band at the top of the column. Both bands were eluted with ethanol after the column had been cut into sections, and the products obtained by evaporation of the solvent. The black band could not be recrystallized and was probably a mixture of decomposition products of the diazonium compound. The yellow band yielded a yellow solid from which light-yellow crystals of diazoaminobenzene were obtained after recrystallization from light petroleum; yield 45%; m.p. 99° (not depressed by an authentic sample of diazoaminobenzene, m.p. 99°). (Found: N, 21·4. Calc. for $C_{12}H_{11}N_3$: N, 21·3%.)

The behaviour of the following compounds was found to be similar to that described for α-alanine. Diazoaminobenzene was isolated in each case by means of the chromatographic method, and identified by means of a mixed m.p. determination. The presence of an aliphatic triazen could not be demonstrated by acidification of the alkaline filtrate.

(ii) *iso*Propylamine (0·34 ml.); yield of diazoaminobenzene 40%.

(iii) β-Aminobutyric acid (0·474 g.); yield 45%.

(iv) DL-Leucine (0·60 g.); yield 25%.

(v) DL-Phenylalanine (0·76 g.); yield 40%. This compound was also allowed to react with benzenediazonium hydrogen sulphate, which was prepared in solid form (Mann & Saunders, 1952) and washed well with ethanol and then with ether to remove impurities. Benzenediazonium hydrogen sulphate (2·5 g.) was dissolved in 10 ml. of ice-cold water and a small quantity of green precipitate removed by filtration. The filtrate was added to 1 g. of phenylalanine dissolved in 40 ml. of cold M-Na_2CO_3 (reaction mixture at pH 10). The mixture was left overnight at 4°. Evolution of N_2 occurred throughout the reaction. The reaction mixture was filtered and the precipitate purified by chromatography on alumina as described above. Diazoaminobenzene was obtained with m.p. 99° (not depressed by an authentic sample, m.p. 99°); yield 45%. The filtrate from the reaction mixture was acidified with 2·5 N-HCl and gave no ppt. The soln. was then extracted with ether several times (total vol. 500 ml.) and the ethereal layer dried with anhydrous $CaSO_4$. Evaporation of the ether yielded a brown syrup which solidified in the presence of light petroleum, b.p. 40–60°. Colourless crystals of DL-phenyllactic acid were obtained after recrystallization from benzene; yield, 43%; m.p. 97° (not depressed by an authentic sample, prepared by a new and simpler method described below). (Found: C, 65·0; H, 6·1. Calc. for $C_9H_{10}O_3$: C, 65·1; H, 6·0%.)

Authentic DL-phenyllactic acid was prepared by dissolving 1 g. of DL-phenylalanine in 50 ml. of water and 4 ml. of conc. HCl; 1·3 g. of $NaNO_2$ in 25 ml. of water was added to the cold soln. and the mixture left overnight at 5°. Extraction of the soln. with ether (200 ml.) and its subsequent distillation yielded a colourless syrup which solidified on rubbing. Colourless crystals of DL-phenyllactic acid were obtained by recrystallization from benzene; yield, 60%; m.p. 97°. (Found: C, 65·1; H, 6·2. Calc. for $C_9H_{10}O$: C, 65·1; H, 6·0%.) Erlenmeyer (1880) gives m.p. 97–98°.

(*c*) *Reactions with miscellaneous compounds.* (i) Methylguanidine (0·483 g. of methylguanidine sulphate). A ppt. was visible after 5 min. and gradually increased during several hours. The product was filtered off, washed with water and dried. Light-yellow crystals of benzenediazomethylguanidine were obtained by recrystallization from benzene; yield 80%; m.p. 89–90° (decomp.). (Found: N, 38·8. $C_8H_{11}N_5$ requires N, 39·5%.)

(ii) 3-Indolylacetic acid (0·826 g.). A yellow precipitate was formed in the initially orange solution after 2 min. Decomposition of this precipitate appeared to take place as a gas was evolved and the yellow precipitate turned brown after 15 min. After standing, the mixture was acidified and the precipitate separated and stirred with 500 ml. of 2·5 N-NaOH, about one-half of the precipitate dissolving. The red solution was filtered and the filtrate acidified with 2·5 N-HCl. A dark-red gelatinous precipitate was obtained. This precipitate was found to decompose on drying to yield a product insoluble in 2·5 N-NaOH. Hence the gelatinous precipitate was dissolved in $CHCl_3$ and reprecipitated from the $CHCl_3$ layer by light petroleum. The product obtained was unstable in hot $CHCl_3$ and benzene, and gave a black syrup. The substance was soluble in 2·5 N-NaOH and dil. aqueous NH_3.

Reaction with diazotized p-*diphenylylamine*

An aqueous soln. of *p*-diphenylyldiazonium chloride was prepared by dissolving 1·5 g. of *p*-diphenylylamine in 50 ml. of hot water and 6 ml. of conc. HCl; the hot solution was filtered and cooled in ice, when *p*-diphenylylamine hydrochloride separated as a white ppt. M-$NaNO_2$ (15 ml.) was

added to the mixture with stirring and the opalescent soln. filtered after standing for 15 min.

Ethanethiol. The above soln. was added to 1·43 ml. of ethanethiol in 50 ml. of cold M-Na_2CO_3. A yellow oil separated immediately and the mixture effervesced vigorously. The oil solidified on stirring to form a yellow solid, which was filtered off, washed with water and dried. Yellow crystals of *p*-diphenylyl ethyl thio ether were obtained by recrystallization from light petroleum, b.p. 40–60°; yield 95%; m.p. 71–72°. (Found: S, 14·9; N, nil. $C_{14}H_{14}S$ requires S, 14·9%.) A sample of the product obtained before recrystallization was dissolved in acetone, filtered and precipitated by the addition of water in the cold. Analysis by the micro-Dumas method showed the absence of nitrogen. Hence the formation of the thio ether was not due to decomposition of the *S*-azo compound during recrystallization from a hot solvent.

Reactions with diazotized p-*arsanilic acid*

An aqueous solution of diazotized *p*-arsanilic acid was prepared by dissolving 1 g. of *p*-arsanilic in 50 ml. of water and 1·5 ml. of conc. HCl; to the ice-cold soln. 23 ml. of 0·2M-$NaNO_2$ was added slowly and the mixture allowed to stand for 15 min.

(*a*) *Reactions in which the product was isolated as an acid.* The following compounds were dissolved in 50 ml. of cold M-Na_2CO_3, the diazo reagent was added and the mixture set aside overnight at 4°.

(i) *n*-Butylamine (0·45 ml.). The yellow–orange soln. was acidified with dil. HCl to yield a yellow ppt. This product was filtered off and purified by dissolving in 2·5N-NaOH and reprecipitating with 2·5N-HCl several times. Yellow crystals of bis-(*p*-benzenearsonic acid diazo)-*n*-butylamine were obtained after drying over conc. H_2SO_4; yield, 60%; decomp. 148°. (Found: N, 13·1; As, 28·2. $C_{16}H_{21}O_6N_5As_2$ requires N, 13·1; As 28·2%.)

(ii) Methylamine (0·318 g. of methylamine hydrochloride). Light-yellow crystals of bis-(*p*-benzenearsonic acid diazo)-methylamine were obtained in a similar manner to that described above; yield 50%; decomp. 137°. (Found: As, 30·4. $C_{13}H_{15}O_6N_5As_2$ requires As, 30·8%.)

(*b*) *Reactions in which the product was isolated as the sodium salt.* The compounds indicated were dissolved in 50 ml. of M-Na_2CO_3, the diazo solution was added and the mixture set aside overnight at 4°. The yellow–orange solutions were acidified with 2·5N-HCl in the presence of *n*-butanol, and the orange butanol layer was separated and shaken with water several times. By adding excess of dil. NaOH (50 ml.; 5%, w/v) the compound was extracted from the butanol in the form of an aqueous solution of the sodium salt. The compound was precipitated by the addition of ethanol (to 80%, v/v) as light-yellow crystals. Some purification was effected by dissolving the crystals in water and reprecipitating with ethanol. The compounds were found to be contaminated with small quantities of Na_2CO_3; hence an indication of the formula of the compounds formed was obtained only by means of the quotient As/N. Approximately 50% yields were obtained.

(i) DL-α-Alanine (0·410 g.). The product corresponds to sodium *pp'*-diazoaminobenzene arsonate. (Found: As/N, 3·61; $C_{12}H_9O_6N_3As_2Na_4$ requires As/N, 3·59.)

(ii) γ-Aminobutyric acid (0·576 g.). The product corresponded to the sodium salt of bis-(*p*-benzenearsonic acid diazo)-γ-aminobutyric acid. (Found: As/N, 2·16. $C_{16}H_{15}O_8N_5As_2Na_5$ requires As/N, 2·14.)

(iii) DL-Phenylalanine (0·76 g.). The product corresponded to sodium *pp'*-diazoaminobenzenearsonate. (Found: As/N, 3·64. $C_{12}H_{19}O_6N_3As_2Na_4$ requires As/N, 3·59.)

The isolation of DL-phenyllactic acid from the reaction of excess of diazo reagent and the amino acid was effected as follows. *p*-Arsanilic acid (1 g.) was dissolved in 15 ml. of N-HCl, the solution cooled and 4·6 ml. of M-$NaNO_2$ added. After standing for 15 min. the soln. was added to 0·25 g. of DL-phenylalanine in 10 ml. of 10% (w/v) Na_2CO_3 and stood for 24 hr. at 4°. The mixture was acidified in the presence of ether (50 ml.) and then extracted a further four times. The ethereal layer yielded a reddish solid which was recrystallized from benzene to give colourless crystals of DL-phenyllactic acid; yield, 90%; m.p. 97° (not depressed by an authentic sample, m.p. 97°).

(*c*) *Reactions with proteins.* Casein (whole) was prepared by the method of Warner (1944). Crystalline bovine-plasma albumin fraction V was obtained from the Armour Laboratories. Edestin was obtained from Roche Products Ltd. Ovalbumin was prepared by the method of Kekwick & Cannon (1936) in the form of a 7·5% (w/v) solution in 0·9% saline and frozen at −20° till required.

The diazo reagent was added to a cold alkaline solution of the protein (see Table 1) in the quantities indicated in the Results section, and the mixture left at 4° overnight. Ethanol was added to an 80% (v/v) concentration and the conjugated protein precipitated by addition of dil. HCl until the pH of the solution was 4·0 as indicated by bromocresol green paper. The ppt. was spun down, dissolved in 25 ml. of 0·5M-$NaHCO_3$, and reprecipitated by addition of 100 ml. of ethanol and 5 ml. of 2·5N-HCl. The final ppt. was dissolved in 25 ml. of 0·5M-$NaHCO_3$ and dialysed for 3 days against 0·5M-$NaHCO_3$. The latter was frequently changed and no coloured materials passed through the cellophan membrane after 24 hr. The recovered solution was precipitated from 80% ethanol as before and the precipitate washed with ethanol, twice with water, twice further with ethanol and finally with ether. The orange–red product was not dried in a desiccator but allowed to equilibrate with the atmosphere for 1 day. The average yield was 50%.

Methods of analysis

Arsenic was determined by the method of Wintersteiner (1926). Nitrogen was determined by a micro-Dumas method by Weiler and Strauss, Oxford. These analyses were accurate to ±1%. Hence the As/N quotient was accurate to ±2%. The Dumas method was used in preference to the Kjeldahl method or that of Friedrich (1933). A typical azo protein gave N = 11·8% by Friedrich's method and N = 10·4% by the Kjeldahl method, compared with N = 12·2% by the Dumas method. Electrophoresis of the conjugated proteins on paper was carried out in an apparatus supplied by Evans Electroselenium Ltd.

Calculation of theoretical As/N *quotients*

The method adopted for the calculation of the As/N quotients which would be expected on the assumption that reaction occurred with various chemical groupings in the protein is an extension of that devised by Hooker & Boyd (1934).

Let the protein contain n different amino acids whose wt./100 g. of protein are $a_1, a_2, \ldots, a_n$. Let the molecular weights of the amino acids be $m_1, m_2, \ldots, m_n$ respectively. Finally, let the free groups of the amino acids combine with

b_1, b_2, ..., b_n moles of diazotized *p*-arsanilic acid/mole of amino acid. Then the wt. (g.) of As combined with 100 g. of protein is

$$75(b_1a_1/m_1 + b_2a_2/m_2 + \ldots + b_na_n/m_n).$$

If this last expression is designated by 75α the N introduced into the protein is (28/75) 75α or 28α. If the wt. of N/100 g. of the untreated protein is M, then the As/N quotient (R) is given by the equation

$$R = \frac{75\alpha}{M + 28\alpha}. \qquad (1)$$

Deamination of α-amino groups may possibly occur, in which case the original N content of the protein is reduced from M to M', where

$$M' = M - 14(a_p/m_p + a_q/m_q \ldots a_z/m_z), \qquad (2)$$

a_p, a_q, ..., a_z being the weights of amino acids present in the form of terminal amino acid residues and m_p, m_q, ..., m_z being their respective molecular weights.

The values for the amino acid content of the proteins used were taken from Tristram (1953). Additional information about the terminal amino acid residues of bovine-plasma albumin was obtained from Vunakis (1951), and of whole casein from Mellon, Korn & Hoover (1953) and Gordon, Semmett, Cable & Morris (1949).

SUMMARY

1. Pauly (1915) has already shown that the phenolic group of tyrosine and the imidazole group of histidine can react with diazo reagents.

2. In a continuation of this work, reaction with other groups present in proteins has been considered and derivatives were prepared with compounds containing certain amino groups and guanidino, sulphydryl and indolyl groups.

3. From the evidence of other authors it is also concluded that imino and aliphatic hydroxyl groups react, whereas all other groups are inert.

4. Of the aliphatic primary amino compounds investigated, only those containing the grouping $-CH_2.NH_2$ have been found to yield triazens. Other types of amino group were deaminated, an aromatic diazoamino compound being produced as the by-product.

5. It is inferred that for amino acids found in proteins, only the ε-amino group of lysine and the α-amino group of glycine are capable of forming bis(diazo)amino links.

6. The maximum amount of diazotized *p*-arsanilic acid which can react with four different proteins has been determined and the values of the arsenic/nitrogen quotients were obtained by analysis.

7. A comparison of these results with theoretical values calculated on the basis of their known amino acid content has given an indication of the extent of the reaction.

8. It is concluded that reaction occurs between one diazonium radical and each sulphydryl and indolyl group; between two diazonium radicals and each phenolic, imidazole, ε-amino group and α-amino group of *N*-terminal glycine residues; and between three diazonium radicals and each guanidino group. It is also concluded that α-amino groups of *N*-terminal residues other than glycine are deaminated.

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β-Carotene in the Flowers of *Narcissus*

By V. H. BOOTH*
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

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Single-flowered plants of the genus *Narcissus* may be divided into three groups. Those in the one group have flowers with yellow perianths and large yellow trumpets; and they come into bloom in Britain in March or April. They are popularly called daffodils, or systematically described as *Narcissus pseudonarcissus*. In another group, popularly called narcissi, of which *Narcissus poeticus recurvus*, or pheasant's eye, is a common example, the flowers have small white perianths and very small pale-yellow cups or coronas fringed with red. These come into flower in May. The middle group of *Narcissus* contains many intermediate types, nearly all of which come into flower in April: they have large white or yellow perianths, and coronas less than half the length of the perianth segments. The coronas may be red, or orange, or white fringed with red. This paper describes an investigation into the nature and the concentration of the red pigment in the coronas of the narcissi in these groups.

EXPERIMENTAL

Material. Several cultivated varieties of *Narcissus* were examined, including some with deep-red coronas grown by Mr J. M. de Navarro. The yellow-trumpet specimens were from commercial varieties of daffodil. The *N. poeticus* flowers were also commercial varieties.

Taxonomy. Attempts were made to use an official taxonomic classification of *Narcissus*, but no advantage was apparent for present purposes. The specimens having red coronas are hybrids derived from various types, predominantly 'large crowned' and ultimately probably *N. poeticus*. Size and shape are dominant criteria for taxonomic classification, whereas deeply pigmented and less-pigmented varieties appear together in more than one class. Therefore it is expedient to use popular names and variety names. The yellow forms of daffodil were derived from *N. pseudonarcissus*.

Methods

Extraction of pigments and spectrophotometry. The pigments were extracted from weighed samples of the coronas into a cold mixture of equal volumes of light petroleum (80–100°) and acetone, saturated with quinol. The acetone was washed from the extract with water, and the light petroleum solution was chromatographed on a mixture of aluminium oxide and sodium sulphate (Booth, 1957). The carotene in solution was measured in the Unicam spectrophotometer with glass prism, model SP. 600. The extinction coefficient ($E^{1\%}_{1cm.}$, at 450 mμ.) for β-carotene in light petroleum was taken as 2550. The total carotenoids, when determined, were measured in the same way before chromatography. Although carotenoids other than carotene mostly have lower E values and are thereby underestimated, this is unimportant in the present application.

RESULTS

Principal pigment from red coronas

Chromatographic behaviour. All the pigments in an extract of the red coronas of *Narcissus* flowers from the intermediate group were adsorbed from light petroleum near the top of the column of alumina. Most of the pigment was eluted by 2% acetone in light petroleum. The colour and behaviour of the most prominent chromatographic band during its elution suggested that the pigment might be β-carotene. The eluate was collected from several extracts treated in this way, acetone was washed from the solution, and the following confirmatory tests were done. Full descriptions of chromatographic technique, animal diet, saponification and other details are described by Booth (1957).

Mixed chromatogram. A portion of the light petroleum solution was mixed with a solution containing a similar quantity of β-carotene prepared in the same way from fresh lucerne (*Medicago sativa*) leaves. The mixed pigment was adsorbed on a long column of alumina and eluted with 2% acetone in light petroleum. Only one chromatographic band was seen, and this exactly resembled a band of β-carotene.

Another portion of this last solution of mixed pigments was chromatographed on magnesia. Again the only visible band resembled that of β-carotene from carrots. No band of α-carotene was seen.

Saponification. A portion of the solution was saponified by shaking with half its volume of methanol plus a half volume of *n*-propanol and 2 vol. of saturated aqueous potassium hydroxide. The pigment was epiphasic (between light petroleum and aqueous diacetone alcohol) both after and before saponification.

* Member of the scientific staff of the Agricultural Research Council.

Crystal form. A portion of the solution prepared from *Narcissus* coronas was concentrated by evaporation under reduced pressure. A few drops of the concentrated solution were placed on a microscope slide and, after the light petroleum had evaporated, the residue was examined under a microscope (480 diameters). The largest crystals were reddish-brown, and the more regular among them were roughly rhombic in shape. They resembled crystals prepared in the same manner from lucerne leaves, and those shown for synthetic all-*trans* β-carotene by Isler, Lindlar, Montavon, Rüegg & Zeller (1956).

Spectral-absorption curve. The pigment from red coronas was adsorbed from light petroleum on to alumina and eluted with 2 % acetone in *n*-hexane. The spectral-absorption curve resembled that of β-carotene from other sources: it had maxima at 451 and 479 mμ. The variety of *Narcissus* used was 'Tantrum'. Another variety, 'Carrowmore', was extracted, and the major pigment gave a similar spectral curve. Both varieties were from the intermediate group.

Rat growth. The carotene-like pigment was extracted from coronas of 'Carrowmore', purified chromatographically, and dissolved in arachis oil. A batch of eight rats, 3½ weeks old, was given a diet free from vitamin A for 3 weeks. Three rats (group C) were given the solution, corresponding in amount to 2·1 μg. as β-carotene, on alternate days. Three other rats (group R) were each given a similar amount of a solution prepared from another variety, 'R 419'. Two rats (group O) received none. Both these control rats developed xerophthalmia, became listless and, after 2 weeks, lost weight. All the dosed rats thrived. The average gains in weight were: group R, 44 g. in 24 days; group C, 40 g. in 24 days; group O, −5 g. in 20 days.

Another eight rats were similarly maintained on the diet free from vitamin A. When they had almost ceased gaining weight, four rats (group RR) were given the second solution, corresponding to 2·1 μg. of β-carotene, on alternate days, and the other four (group OO) were given none. In 12 days group RR gained an average of 28 ± 2·3 g. and group OO lost 17 g.

Liver storage. Two of the RR rats were then given all that remained of the two solutions, corresponding to 53 μg. of β-carotene/rat. Their livers were removed, digested with potassium hydroxide, and extracted with diethyl ether (Davies, 1933). The ether was evaporated, and the residue dissolved in chloroform and treated with antimony trichloride reagent. From the extinction of the blue colour, determined in an absorptiometer, it was calculated that the livers had 29 and 38 i.u. of vitamin A. No vitamin A was found in the livers of two undosed rats from group OO.

Carotenoid contents of coronas

Carotene. The major pigment in the coronas of narcissi in the intermediate group was estimated on the assumption that it was β-carotene. Varieties examined included 'Brodie 132/F/30', 'Carrowmore', 'Firecracker', 'Firemaster', 'Limerick', 'Mahmoud', 'Matapan', 'Scarlet Elegance' and 'Tantrum'. The β-carotene contents of the specimens of coronas ranged from 1·1 to 3·1 mg./g. based on fresh weight. Dry weights, determined by weighing before and after heating at 100° for 4 hr., of these and other varieties ranged from 11·8 to 16·5 %, average 13·9. The sample having the highest carotene content had dry matter 15·2 %; hence its β-carotene content on a dry-weight basis was 20·4 mg./g.

The flowers of the varieties named above had red coronas. Samples of other varieties having orange or deep-yellow coronas were also analysed. These had β-carotene contents of from 0·15 to 0·9 mg./g. based on fresh weight; they included 'Flamenco', 'Gala', 'Kilworth', 'Monique' and others whose names were not known.

The carotene values are not listed because it is doubtful whether the specimens examined were representative, for the following reasons. (1) The pigment in *Narcissus* coronas is bleached by sunlight, particularly in richer varieties; (2) the carotene content may vary with the maturity and growing conditions as it does in the carrot (Booth & Dark, 1949); (3) only small samples were examined.

Physical state of carotene. Sections of *Narcissus* coronas from the intermediate group were mounted in water and examined under a microscope (1000 diameters). Large numbers of long, roughly rectangular, yellow crystals were visible.

Carotenols. Other yellow fat-soluble pigments, presumably hydroxycarotenoids, that could be eluted from alumina by polar solvents, beginning with 5 % acetone in light petroleum, were also present in the extracts of *Narcissus* coronas. They were colorimetrically equivalent to almost half the β-carotene content. About half of this carotenol fraction was readily eluted in a manner strongly suggesting that it was in the esterified form. The higher concentrations of acetone that were necessary to elute them, and the complexity of their chromatographic bands, suggested that the other pigments comprised a variety of carotenoids. When a diethyl ether extract of coronas was treated with concentrated hydrochloric acid, no green colour was produced. Evidently furanoid epoxides were not present in appreciable amounts.

Pigments in perianth. The perianths of the flowers having deepest red coronas were usually white; but some of the varieties having red coronas had yellow perianths. The bulk of the pigments extracted from

yellow perianths were held on alumina in the manner of carotenols, and only a trace of carotene was present. No green colour was seen when a solution of the pigments in diethyl ether was treated with concentrated hydrochloric acid.

Pheasant's eye narcissus

The coronas or cups of flowers of the pheasant's eye type have deep-red fringes less than 1 mm. wide. The fringes from several flowers of *N. poeticus recurvus* were cut off and extracted. Most of the pigment behaved chromatographically like β-carotene. The mean yield from three samples of different origin was 51·5 mg./g. Since the dry weight was 31·1 %, the major pigment formed 16·6 % of the dry weight.

The pigment from *N. poeticus* was not studied in such detail as that from the coronas of flowers in the intermediate group. However, the chromatographic behaviour, the colour of the chromatographic band, the colour of the solution, the resistance to saponification, the shape and colour of the crystals and the position of the spectral bands in the visible region resembled those of β-carotene. The shape of the spectral absorption curve between 280 and 380 mμ. closely resembled that for all-*trans* β-carotene shown by Zechmeister (1944).

Yellow daffodils

Carotenoid content. The yellow coronas or trumpets from flowers of the *N. pseudo-narcissus* type, having yellow perianths, were similarly extracted, and the light petroleum solution was chromatographed on a mixture of alumina and sodium sulphate. The greater part of the pigment was not eluted by 2 % acetone in light petroleum. The major pigment was therefore not carotene, but its behaviour was consistent with its being carotenoid in nature. The trumpets had a total carotenoid content of about 0·87 mg./g. and a β-carotene content of 0·013 mg./g. Thus in carotenol content these yellow trumpets resembled red coronas, but their carotene content was of a different order altogether. The dry matter, determined as before, was 11·8 %. Hence the β-carotene content on a dry-weight basis was 0·11 mg./g.

A sample of another variety of daffodil having very large trumpets (5 cm. long) was examined. The total carotenoid content of the trumpets was 0·22 mg./g., but the carotene content was only 0·004 mg./g.

When diethyl ether extracts of the daffodil trumpets (both 5 and 3½ cm. types) were treated with concentrated hydrochloric acid, a green colour developed slowly. Evidently the daffodil pigments included epoxides.

Leaves

Leaves of *Narcissus*, from each group taken at time of full bloom, had an average carotene content of 0·056 mg./g. based on fresh weight.

DISCUSSION

Courchet (1888), Bidgood (1905) and van Wisselingh (1914) reported the presence of carotenoids in flowers of *Narcissus*, and Coward (1923) found that the flowers supported growth in vitamin A-deficient rats. Hence it could be inferred from this early qualitative work that *Narcissus* blooms contain a carotenoid having vitamin A activity, but no estimates of its concentration have been found in the literature.

The identity tests described in this paper (colour in solution, chromatographic appearance, mixed chromatogram, resistance to saponification, crystal form, spectral-absorption curve and biological response) taken together provide good evidence for the hypothesis that the major component of the red pigment in *Narcissus* coronas is all-*trans* β-carotene. Although sixteen rats are too few for assaying biological activity, it is improbable that any pigment other than β-carotene would give so high a response; for, while ten rats thrived on 1·05 μg./day, all the undosed rats declined.

The coronas of *Narcissus* represent one of the most concentrated sources of carotene in Nature. Coronas from the intermediate group had carotene contents ranging from 0·15 to over 3 mg./g., and upon microscopic examination of the coronas, crystals of carotene were clearly seen. In the richest specimens the carotene comprised 2 % of the dry weight. Some of the most deeply coloured varieties are noteworthy for fading in sunlight. Therefore it is unlikely that the carotene was determined while it was at maximal concentration, and, if more specimens and more varieties were examined, doubtless even higher values would be found. In the fringes from the coronas of *N. poeticus recurvus* the average concentration was 16·5 % of the total dry matter, and in addition other carotenoids were present with the carotene. It is noteworthy that the dry-matter content of these fringes was higher than that of coronas from other types of *Narcissus*. These very high concentrations of carotene suggest that this material might be useful in studies on carotenogenesis.

The pigments in the trumpets of the yellow daffodil, although presumably also carotenoids, only included a small percentage of β-carotene, thereby differing from the *Narcissus* type. The carotene contents of *Narcissus* leaves were only of the same order as those of other coarse leaves.

SUMMARY

1. The major pigment in the red coronas of certain cultivated varieties of *Narcissus* was separated from other pigments chromatographically on alumina.

2. The unknown pigment showed a chromatographic behaviour like that of β-carotene, and it could not be separated from β-carotene on alumina or magnesia. The spectral-absorption curve resembled that of β-carotene. In colour and shape the crystals resembled those of all-*trans* β-carotene. The pigment supported growth in rats deficient in vitamin A, and led to storage in their livers of a substance that gave a blue colour with antimony trichloride reagent. It is concluded that the pigment is β-carotene.

3. The carotene was found in the coronas in concentrations up to 3 mg./g. based on the fresh weight, or 2% of the dry weight. In red fringes only from the coronas of *N. poeticus recurvus* the carotene comprised about 16·5% of the dry weight.

4. Other pigments, probably free and esterified carotenols, were present in amounts approximately equivalent to half the β-carotene contents.

5. Pigments from trumpets of yellow forms ('daffodil'), although presumably carotenoids, included only small proportions of carotene.

6. Tests with hydrochloric acid suggested that the trumpets of yellow forms contained epoxides but that the coronas and perianths of the red-corona forms did not.

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Steroids of Pregnant Mare's Urine

5. IDENTIFICATION OF TWO ANDROSTANE-3:16-DIOLS. THE STRUCTURE OF 'URANOLONE'*

By R. V. BROOKS† and W. KLYNE
Postgraduate Medical School, London, W. 12

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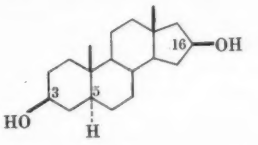
Brooks, Klyne, Miller & Paterson (1952) described the fractionation of the neutral non-ketonic lipids of pregnant mare's urine and the isolation of a number of compounds, some of which were not identified. The main purpose of the present paper is to report the identification of two of these unknown compounds (*MN* and *MM*) as the 5α-androstane-3β:16α- and -3β:16β-diols. A preliminary account of this work has already been given (Brooks & Klyne, 1956).

The structure of 'uranolone' (Marker, Lawson, Wittle & Crooks, 1938*b*) has been confirmed.

Identification of 5α-androstane-3β:16α- and -3β:16β-diols

The compounds *MM* and *MN* were obtained as their benzoates by the methods described by Brooks *et al.* (1952). Comparison of the properties of *MM* and its derivatives with those quoted by Fajkoš & Šorm (1955) for 5α-androstane-3β:16β-diol (I; cf. also Huffman & Lott, 1955) suggested that the two substances might be identical. Comparison of our materials with authentic samples supplied by Professor Šorm proved the identity conclusively (see Table 1, p. 665).

In the structural formulae of the steroids in this paper the convention followed by the Chemical Society has been used, i.e. interrupted lines represent α-configuration and bold continuous lines β-configuration.



(I)

5α-Androstane-3β:16β-diol

* Part 4. Brooks, Klyne, Miller & Paterson (1952).

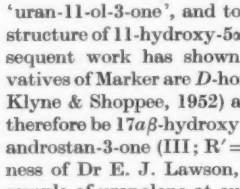
† Present address: St Thomas's Hospital Medical School, London, S.E. 1.

The establishment of the identity of *MM* as the 3β:16β-diol led us to consider whether one of our other unknown compounds might be the 3β:16α-epimer. Two benzoates, those of compounds *MN* and *MS*, had melting points close to that of 5α-androstane-3β:16α-diol dibenzoate. Comparison of the infrared spectra of these materials and mixed m.p. determinations showed clearly that *MN*, and not *MS*, was identical with the 3β:16α-diol, again supplied by Professor Šorm (Table 1).

This identity was confirmed by hydrolysis of *MN* dibenzoate to the diol and subsequent preparation of the diacetate; elementary analyses and determination of optical rotations were not carried out in this series, because only 4 mg. of dibenzoate was available for the hydrolysis and acetylation.

'*Uranolone*'

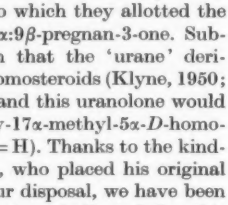
Marker *et al.* (1938*b*) isolated from pregnant mare's urine a hydroxy ketone which they called 'uran-11-ol-3-one', and to which they allotted the structure of 11-hydroxy-5α:9β-pregnan-3-one. Subsequent work has shown that the 'urane' derivatives of Marker are *D*-homosteroids (Klyne, 1950; Klyne & Shoppee, 1952) and this uranolone would therefore be 17*aβ*-hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one (III; R′ = H). Thanks to the kindness of Dr E. J. Lawson, who placed his original sample of uranolone at our disposal, we have been able to confirm the structure indicated for this compound. This is of interest since 3-oxo steroids are not of common occurrence in urine.



(II)

'Uranediol'

17α-Methyl-5α-*D*-homoandrostane-3β:17*aβ*-diol



(III)

'Uranolone'

17*aβ*-Hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one

The preparation of the hydroxy ketone (III; as its benzoate, R′ = Bz) was facilitated by a chance finding, that treatment of the dibenzoate of uranediol (17α-methyl-5α-*D*-homoandrostane-3β:17*aβ*-diol (II; R = R′ = Bz) with potassium carbonate in aqueous methanol hydrolysed only the 3β-benzoyloxy group, yielding the 17*aβ*-monobenzoate (II; R = H; R′ = Bz). Oxidation of the monobenzoate with chromium trioxide in acetic acid yielded the benzoyloxyketone (III; R′ = Bz), which was identical with the benzoate of Lawson's compound (Table 2, p. 665).

The presence of the keto group at C-3 in (III) was demonstrated by the dinitrobenzene reaction (Zimmermann, 1935, 1936; Callow, Callow & Emmens, 1938; cf. also Marker *et al.* 1938*b*); the absorption spectra of the colours obtained by reaction for 5 and 60 min. were almost identical with those given by 5α-cholestan-3-one (Broadbent & Klyne, 1954). The other hydroxy ketone derived from uranediol, i.e. 3β-hydroxy-17α-methyl-5α-*D*-homoandrostan-17*a*-one, gives no colour in the dinitrobenzene reaction, because it has no free methylene group adjacent to the carbonyl at C-17*a*. (This compound has recently been prepared by reduction of uranedione with sodium borohydride; W. Klyne, unpublished observations.)

The partial hydrolysis of the dibenzoate (II; R = R′ = Bz), in which the 17*aβ*-benzoyloxy group is resistant to alkali, provides an interesting example of steric hindrance by adjacent methyl groups on both sides of the benzoyloxy group, although this is most probably equatorial (cf. Brooks *et al.* 1952). The hindered benzoate group of the compound (III; R′ = Bz) was hydrolysed to a considerable extent by refluxing with 8% (w/v) potassium hydroxide in aqueous methanol for 9 hr.

EXPERIMENTAL

General methods

Methods for melting points (which are corrected), specific rotations, microanalyses, chromatograms on alumina, 'usual working up' and preparation of acetates and benzoates were as described by Brooks *et al.* (1952). Ultraviolet absorption spectra were determined in ethanol with either a Beckman (DU) or a Unicam (SP. 500) spectrophotometer. Alumina for chromatography was of Activity II (Brockmann & Schodder, 1941).

Infrared absorption spectra were measured in a Grubb-Parsons S3 A single-beam instrument between 1200 and 900 cm.$^{-1}$ (the 'finger-print' region) in CS_2 or in $CHCl_3$.

Identification of compounds MN *and* MM *as* 5α-*androstane*-3β:16α- (*and* -3β:16β-*diols*

The properties of the two urinary compounds (Brooks *et al.* 1952) are compared with those of the authentic 3:16-diols (Fajkoš & Šorm, 1955) in Table 1.

Urane derivatives

17α-*Methyl*-5α-D-*homoandrostane*-3β:17a*β*-*diol* 17a-*monobenzoate* (*II*; R = H, R′ = Bz; *uranediol* 17a-*monobenzoate*). Uranediol dibenzoate (32 mg., m.p. 212–213°) in methanol (10 ml.) was treated with potassium carbonate (140 mg.) in water (1·4 ml.) and refluxed for 5 hr. The product (29 mg.) was obtained by the usual working up (extraction with ether; washing with dilute alkali, dilute acid and water; drying with Na_2SO_4); it was chromatographed on Al_2O_3 (1·8 g.). Light petroleum–benzene mixture (1:3, v/v) and benzene eluted 23 mg. of material, m.p. 213–217°. Recrystallization of this from methylene chloride–methanol yielded the 17a-*monobenzoate* as plates, m.p. 219–220°; this

Table 1. *Identification of compounds* MM *and* MN *as* 5α-*androstane*-3β:16-*diols*

	Compound *MM*		
	MM		5α-Androstane-3β:16β-diol*
Diol			
M.p.	180–182°		182–183°†
Mixed m.p.		180–182°	
$[\alpha]_D$ (EtOH)	−6°		−6°
Analysis	C, 78·3; H, 10·9%		C, 78·1; H, 10·9%‡
Infrared spectra ($CHCl_3$)		Identical	
Diacetate			
M.p.	108–109°		105–107°
Mixed m.p.		105–107°	
$[\alpha]_D$ ($CHCl_3$)	−14°		−8°
Infrared spectra (CS_2)		Identical	
Dibenzoate			
M.p.	188–190°		188–190°
Mixed m.p.		188–190°	
$[\alpha]_D$ ($CHCl_3$)	−15°		−14°
Analysis	C, 78·8; H, 8·2%		C, 79·2; H, 8·1%
Infrared spectra (CS_2)		Identical	
	Compound *MN*		
	MN		5α-Androstane-3β:16α-diol*
Diol			
M.p.	186–187°		186–187°
Mixed m.p.		186–188°	
Diacetate			
M.p.	172–173°		172–173°
Mixed m.p.		173–175°	
Infrared spectra (CS_2)		Identical	
Dibenzoate			
M.p.	183–184°		184–185°
Mixed m.p.		183–184°	
Infrared spectra (CS_2)		Identical	

* Samples from Professor F. Šorm; m.p.'s determined in this Laboratory; values for $[\alpha]_D$ are those given by Fajkoš & Šorm (1955).

† A sample of this compound from Dr M. N. Huffman had m.p. 182–184°; mixed m.p. with compound *MM*, 182–183°.

‡ Analysis values in this column are calculated for $C_{19}H_{32}O_2$ and $C_{33}H_{40}O_4$ respectively.

compound does not sublime before melting—contrast uranediol itself. $[\alpha]_D^{20}$ +21 in $CHCl_3$ (c = 0·8), molecular rotation (M_D), +91. Ultraviolet absorption (in ethanol) $\lambda_{max.}$ 230 and 273 mμ.; log $\epsilon_{max.}$ 4·16 and 2·98 respectively. (Found: C, 79·0; H, 9·5; $C_{28}H_{40}O_3$ requires C, 79·2; H, 9·5%.)

17α*β-Benzoyloxy*-17α-*methyl*-5α-D-*homoandrostan*-3-*one* (*III*; R′ = Bz; *uranolone benzoate*). Uranediol monobenzoate (II; R = H, R′ = Bz; impure, containing some free uranediol: 84 mg.) was dissolved in acetic acid (20 ml.); chromium trioxide (0·2 M in acetic acid; 2 ml.) was added, and the mixture was allowed to stand overnight at room temperature. The neutral product (71 mg.) was isolated by extraction with ether, washing with dilute alkali, dilute acid and water, and drying (Na_2SO_4); it was chromatographed on Al_2O_3 (5 g.). Light petroleum–benzene mixtures (1:1 and 1:3, v/v) eluted 43 mg. of the benzoyloxyketone. Benzene and benzene–ether mixtures eluted small quantities of material which had infrared spectra very similar to that of uranedione.

The *benzoyloxyketone*, after recrystallization from methanol containing a little $CHCl_3$, formed leaflets, m.p. 239–241°. $[\alpha]_D^{20}$ +60 in $CHCl_3$ (c = 0·7), $[M]_D$ +254. Ultraviolet absorption (in ethanol) $\lambda_{max.}$ 230 and 272 mμ.; log $\epsilon_{max.}$ 4·10 and 2·76 respectively. (Found: C, 79·5; H, 9·2. $C_{33}H_{40}O_3$ requires C, 79·0; H, 9·3%.) For comparison with the benzoate of Lawson's uranolone see Table 2.

The dinitrobenzene reaction (Callow *et al.* 1938), carried out according to Broadbent & Klyne (1954) for periods of 5 min. and 60 min. on the benzoyloxyketone, gave absorption spectra (Fig. 1) which were almost identical with those given by 5α-cholestan-3-one.

17α*β-Hydroxy*-17α-*methyl*-5α-D-*homoandrostan*-3-*one* (*III*; R′ = H; *uranolone*). The benzoate (IV; R′ = Bz; 12 mg.) was dissolved in 5 ml. of boiling methanol; KOH (400 mg.) in water (0·8 ml.) was added and mixture was heated under reflux for 9 hr. The product obtained by the

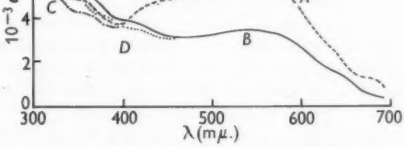


Fig. 1. Absorption spectra of colours produced in Zimmermann dinitrobenzene reactions. Uranolone benzoate, *A* (- - -), 5 min.; *B* (—), 60 min.; 5α-Cholestan-3-one, *C* (-·-·-), 5 min.; *D* (. . . .), 60 min. Curves *C* and *D* are almost superposable on *A* and *B* respectively above 400–450 mμ.

Table 2. *Comparison of uranolone and* 17α*β-hydroxy*-17α-*methyl*-5α-D-*homoandrostan*-3-*one*

	Uranolone*		17α*β*-Hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one
Hydroxy compound (III; R′ = H)			
M.p.	162–165°		167–169°
Mixed m.p.		165–170°	
Infrared spectrum (in CS_2)		Identical	
Benzoate (III; R′ = Bz)			
M.p.	234–237°		237–239°
Mixed m.p.		236–240°	
Infrared spectrum (in CS_2)		Identical	

* Sample provided by Dr E. J. Lawson.

usual working-up (extraction with ether; washing with dilute alkali, dilute acid and water; drying with Na_2SO_4) was chromatographed on Al_2O_3 (1 g.) and the infrared spectra of selected fractions were examined for the presence of the characteristic benzoate band at about 1110 cm.$^{-1}$. The first four fractions obtained with light petroleum–benzene (1:3, v/v) contained 1 mg. of material (strong benzoate bands); the second four fractions with the same eluent contained 3 mg., m.p. 164–169° (faint benzoate bands). The last three fractions with the same eluent and five fractions with benzene (3 mg.) had m.p. 165–169° and showed no benzoate bands. Recrystallization from acetone yielded crystals, m.p. 167–169°. For comparison with Lawson's uranolone see Table 2.

DISCUSSION

The present work emphasizes once again the important species differences which may occur in the nature of the urinary steroids between mammals in pregnancy. The only two species hitherto investigated thoroughly are the human female and the mare. (For reviews see Pearlman, 1948; Hirschmann, 1955.) Some work on two other species—goat and cow—has been presented briefly (Wright & Klyne, 1955; Klyne & Wright, 1956*a*, *b*).

The differences in steroid content between human and mare's pregnancy urine may be considered under the following headings.

Phenolic steroids. Human urine contains only *A*-ring aromatic steroids; mare's urine contains considerable quantities of compounds with additional double bonds in the *B* ring (equilin) or with both *A* and *B* rings aromatic (equilenin).

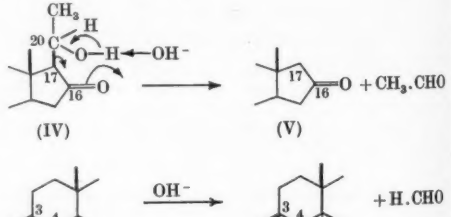
Pregnanediol isomers. Although human urine contains a great preponderance of one isomer, 5β-pregnane-3α:20α-diol, mare's urine contains considerable quantities of two isomers with the common steroid skeleton (5α-pregnane-3β:20α- and -3β:20β-diols), of which the latter seems to preponderate towards the end of pregnancy. Mare's urine contains in addition the *D*-homosteroid uranediol, and, in smaller quantity, a number of other constituents, which are probably pregnanediol or androstanediol isomers.

Urane derivatives. There is no mention in the literature of the occurrence of these compounds in any natural source but mare's urine—except for one claim by Marker, Kamm, Oakwood, Wittle & Lawson (1938*a*) to have obtained uranetrione by oxidation of a fraction from human pregnancy urine, and a statement by Dobriner (1952) that small quantities of uranediol (identified by infrared spectra) had been isolated from the same source. Although many schools have worked intensively on human pregnancy urine for years, these findings have not been confirmed. Fukushima & Gallagher (1956) have discussed the identification of some *D*-homosteroids obtained from extracts of human urine; these, as they say, are most probably artifacts.

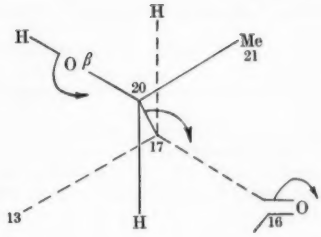
The suggestion has been made (Klyne, 1953) that urane derivatives might arise in the mare by rearrangement *in vivo* of 17α:20β-diols to 17-methyl-*D*-homoandrostan-17*a*-ones, followed by reduction; this hypothesis was based on theoretical considerations regarding the preferred conformation of the side chain. Some support is lent to this idea: first, by the finding in this Laboratory (Brooks, unpublished work) that the ratio of 3β:20β to 3β:20α isomers is much higher in late-pregnancy than in mid-pregnancy urine (uranediol has been isolated by us only from late-pregnancy urine); secondly, by the work of Ramirez & Stafiej (1955*a*, *b*, 1956), who have shown that the rearrangement of 20ξ-amino-17ξ-hydroxy compounds to *D*-homo ketones *in vitro* is strictly stereospecific in the way suggested for 17:20-diols by Klyne.

16-*Substituted androstanes.* The existence of a compound now identified as 3β-hydroxy-5α-androstan-16-one in pregnant mare's urine has been known for some years (Heard & McKay, 1939; Oppenauer, 1941), although its structure was not proved until recently (Huffman & Lott, 1951). The two 3β:16-diols reported in this paper are clearly related to Heard's hydroxy ketone. Such 3:16-disubstituted androstanes have not been reported from any other natural sources, although 5α-pregnane-3β:16α:20β-triol (Haslewood, Marrian & Smith, 1934; Hirschmann, Hirschmann & Daus, 1949) is one of the major constituents of the triol fraction from mare's urine, and 3:16:17-triols (including oestriol) are well-known constituents of human urine (for references see Hirschmann, 1955; Marrian & Bauld, 1955).

The 5α-androst-16-en-3α-ol of Brooksbank & Haslewood (1950) may of course be considered as an *in vivo* dehydration product of either a 16-hydroxy or a 17-hydroxy compound.

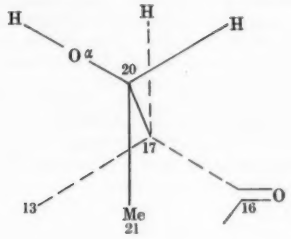


A plausible mechanism for the formation of the 3:16-disubstituted androstanes without a C-17 substituent *in vivo* is the following. Any process involving side-chain oxidation must necessarily give a 17-hydroxy or 17-oxo compound; a hydrolytic



(VIII)

20β-Hydroxy-16-one



(IX)

20α-Hydroxy-16-one

(Perspective views looking down C-20—C-17 bond.)

process of some kind must therefore be considered. A 20-hydroxypregnan-16-one (as IV) is a β-hydroxy ketone and might undergo a reverse aldol condensation to give a 16-oxoandrostane (V); an analogy *in vitro* is provided by the ready formation of formaldehyde by the action of alkali on the triterpene icterogenin (VI), yielding a 24-nortriterpene (VII) (Barton & de Mayo, 1954).

If the 16-one (V) does arise by a process like that suggested above, the 20β-hydroxy-16-one should react more readily than its 20α-epimer for the following reason. Only the 20β-compound can easily take up the side-chain conformation (VIII) required for a concerted reaction, in which C-16, C-17, C-20 and O-20β are coplanar and the bonds 16–17 and 20–O are antiparallel [cf. formula (XX) in Klyne, 1953]; here the hydrogen atom at C-20 is close to the angle methyl group at C-13.

For the 20α-hydroxy isomer, the similar conformation (IX) is unfavourable, because here the methyl group at C-20 (C-21) would be close to the angle methyl group and subject to steric strain.

SUMMARY

1. Two neutral non-ketonic steroids of pregnant mare's urine (compounds *MN* and *MM* of Brooks *et al.* 1952) have been identified as the 5α-androstane-3β:16α- (and -3β:16β-)diols.

2. The constitution of 'uranolone' (Marker *et al.* 1938*b*) has been confirmed as 17*aβ*-hydroxy-17α-methyl-5α-*D*-homoandrostan-3-one.

3. Species differences in urinary steroids between the human female and the mare are discussed.

We are indebted to the Agricultural Research Council for grants for expenses which have largely defrayed the costs of this work, and for a personal grant to one of us (R. V. B.). We are most grateful to Ayerst, McKenna and Harrison (Montreal), British Drug Houses Ltd. (London), N. V. Organon (Oss, Netherlands) and Ovaltine Laboratories (King's Langley, Herts) for their generous help in the provision of mare's urine; Professor F. Šorm (Czechoslovak Academy of Science, Prague) and Professor M. N. Huffman (Oklahoma Medical Research Foundation) for reference compounds; Dr E. J. Lawson (Sterling–Winthrop Research Institute, Rensselaer, N.Y.; formerly of Pennsylvania State College) for putting at our disposal his original sample of uranolone; Dr I. D. P. Wootton, Mrs P. Bane and Miss Sylvia Saunders for the infrared spectra; and Miss Miriam Mattock for much technical assistance in the preparation of urane derivatives.

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Studies on Sulphatases

15. THE ARYLSULPHATASES OF HUMAN SERUM AND URINE*

By K. S. DODGSON and B. SPENCER
Department of Biochemistry, University of Wales, Newport Road, Cardiff

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Conflicting reports concerning the arylsulphatase activity of serum and urine have appeared in the literature. Thus Pantlischko & Kaiser (1952), using phenolphthalein disulphate as substrate, could detect no arylsulphatase activity in urine, whereas Russo (1947), using phenyl sulphate, stated that the arylsulphatase activity of urine towards this substrate was quantitatively comparable with that of animal tissues excluding liver. Huggins & Smith (1947) found activity equivalent to the liberation of 0·3–15·0 and 0·9–19·7 μg. of *p*-nitrophenol/hr./ml. from *p*-nitrophenyl sulphate (NPS) by serum and urine respectively, and Abbott & East (1949), using the same substrate, observed increased serum arylsulphatase activity after induced liver damage in the rat. On the other hand, Abbott (1947) could detect no hydrolysis of phenyl sulphate by human serum over a period of 18 hr. Dodgson & Spencer (1954) were unable to confirm the observations of Huggins & Smith (1947), the activities recorded corresponding to the release of only 0–0·2 μg. of *p*-nitrophenol/hr./ml. by either urine or serum. More recently Boyland, Wallace & Williams (1955) used potassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) as the assay substrate, and reported that the arylsulphatase activities of normal urine and serum were respectively 0–0·82 and 1·2–3·3 μg. of nitrocatechol liberated/hr./ml. These authors observed increased activity in several infected urines and in urines from patients with carcinoma of the bladder and other sites and from patients with tuberculosis. There was no parallel rise in serum arylsulphatase activity.

Recently, the arylsulphatase activity of mammalian tissues has been shown to be due to three separable enzymes which can be distinguished by their relative substrate affinities, behaviour towards inhibitors and ease of solubilization (Dodgson, Spencer & Thomas, 1955; Dodgson, Spencer & Wynn, 1956). Two of the enzymes (arylsulphatases *A* and *B*) are easily obtained in solution from mammalian livers after rupture of the mitochondria of the liver cells (cf. Viala & Gianetto, 1955) and have been called 'soluble' or mitochondrial arylsulphatases (Dodgson *et al.* 1955, 1956). They have a high affinity and activity towards NCS but have little affinity or activity towards NPS or potassium *p*-acetylphenyl sulphate (APS). The substrate specificity of the other arylsulphatase (arylsulphatase *C*) is the converse of that shown by the 'soluble' enzymes. This enzyme is found exclusively in the microsomes of the liver cell (Dodgson, Spencer & Thomas, 1954, 1955) and has been obtained in a soluble form only after treatment with crude pancreatic extracts in the presence of a non-ionic surface-active agent (Dodgson, Rose & Spencer, unpublished results).

A re-assessment of the urinary and serum arylsulphatase patterns has now been made in the light of these findings.

* Part 14: Dodgson, Lloyd & Spencer (1957).

EXPERIMENTAL

Serum and urine samples. Blood samples from humans, dogs and rabbits were collected intravenously and from rats by cardiac puncture. Ox and sheep bloods were fresh slaughter-house specimens. Whenever possible sera were separated and assayed within 1 hr. of collection of the blood. Urines were assayed immediately after voiding.

Assay of arylsulphatase activity. The substrates APS, NPS and NCS were prepared as previously described (Dodgson *et al.* 1955). No attempt was made to purify the NCS

further, as recently suggested by Dodgson & Spencer (1956*a*). The method of determination of the enzyme activity of human-liver preparations by spectrophotometric measurement of the phenols liberated from these substrates (Dodgson *et al.* 1955) was adapted to the low activities found in urine and serum. To 0·4 ml. of the substrate, dissolved in 0·5 M sodium acetate–acetic acid buffer at the required pH and contained in a 15 ml. tapered centrifuge tube, was added 0·4 ml. of serum or urine which had been adjusted to the same pH with acetic acid or NaOH. After incubation at 38° for the required period, 2 ml. of 0·2 N-NaOH was added for APS and NPS and 2 ml. of alkaline quinol mixture for NCS. When NCS was used as the substrate the alkaline quinol mixture was made up immediately before use according to the directions of Roy (1953), except that the $Na_2SO_3,7H_2O$ was dissolved in 0·5 N-NaOH. The tubes were centrifuged at 2500 *g* for 10 min. and spectrophotometric readings at 323 mμ. (APS), 400 mμ. (NPS) and 515 mμ. (NCS) were made within 30 min. with serum or at leisure with urine. All assays were carried out in duplicate with duplicate controls in which serum or urine and the buffered substrate solution were incubated separately and mixed immediately before the addition of alkali. The amounts of the phenols liberated were calculated as follows, the appropriate molecular extinction coefficient being used (see Dodgson & Spencer, 1953):

$$\mu\text{g. of phenol liberated/hr./ml. of urine or serum} = \frac{(E_t - E_c) \times \text{mol.wt.} \times 2{\cdot}8 \times 10^6}{T \times \epsilon_{max.} \times 0{\cdot}4 \times 10^3},$$

or

$$= \frac{(E_t - E_c) \times F}{T},$$

where E_t and E_c are the $\log_{10} I_0/I$ readings of the test and control determinations respectively, and F is a factor which is 47·6 for *p*-hydroxyacetophenone, 53·5 for *p*-nitrophenol and 96·06 for 4-nitrocatechol; T is the time of incubation in hr. and was usually 18–24 hr. for APS and NPS and 2 hr. for NCS.

An APS, NPS or NCS unit of arylsulphatase activity is defined as that which liberates 1 μg. of the respective phenol in 1 hr. under the stated conditions.

Non-enzymic hydrolysis of NPS by serum at alkaline pH. In the assay of serum with NPS, N-NaOH was originally used to stop enzyme action and to convert the liberated *p*-nitrophenol into the anionic form ($\lambda_{max.}$ 400 mμ.). However, under these conditions an increase in optical density at 400 mμ. occurred on allowing the final alkaline mixture to stand. It can be seen from Fig. 1 that this increase was due to three factors: the development of turbidity in the mixture, a slow liberation of *p*-nitrophenol owing to the alkaline hydrolysis of NPS (cf. Burkhardt & Wood, 1929) and hydrolysis of NPS by a reaction catalysed by serum at alkaline pH. The onset of the serum-catalysed reaction and the increase in turbidity were both preceded by an initial lag period (see Fig. 1) the duration of which was greatly extended by decreasing the concentration of NaOH or by lowering the temperature. By using 0·2 N-NaOH in the assay method the duration of the lag period was so lengthened that the increase in optical density at 400 mμ. was negligible over 1 hr. Increasing the temperature or the concentration of NaOH not only decreased the duration of the lag phase but also increased the rates of turbidity formation and of *p*-nitrophenol liberation. The lag phase could be eliminated by pre-incubating the serum with alkali before addition of the NPS, the time required being dependent on temperature and the concentration of alkali used.

The sera and plasma of man, ox, dog, sheep, rat and rabbit possessed the NPS-hydrolysis factor. The factor was heat-stable and non-dialysable and did not catalyse the hydrolysis of APS, NCS, potassium *m*-nitrophenyl sulphate or phenolphthalein disulphate. Both the albumin and globulin fractions of serum were capable of catalysing the hydrolysis although albumin appeared to be the more active of the two. Later the catalysis was seen to be a property of many proteins, although high concentrations were usually necessary before the effect was shown. It seemed possible that the ability of proteins to catalyse the alkaline hydrolysis of NPS might depend on the presence of certain amino acids. Of a series of amino acids tested, cystine, cysteine, methionine, proline and hydroxyproline were capable of catalysing the liberation of *p*-nitrophenol from NPS in the presence of N-NaOH.

A similar phenomenon, the alkaline hydrolysis of *p*-nitrophenyl acetate, phosphate and benzoate catalysed by albumin and sulphur-containing amino acids, has been observed by Perenyi (1954*a–c*). Perenyi examined the reactions in detail and proposed a general reaction mechanism which involved the SH groups present in the protein or the amino acids. The lag phase observed with serum was thought to be due to the gradual release of SH groups by alkali. It is possible that a similar mechanism might explain

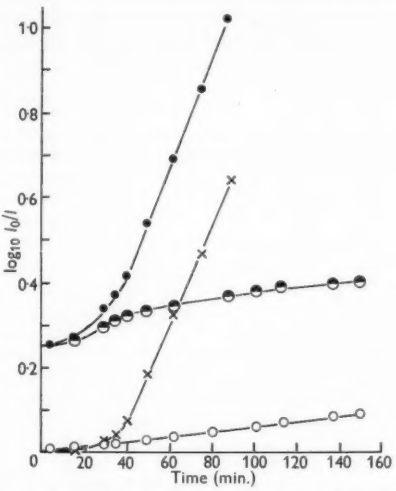

Fig. 1. The effect of NaOH on the turbidity of serum and the serum-catalysed liberation of *p*-nitrophenol from NPS. ●, Increase in the $\log_{10} I_0/I$ value at 400 mμ. for a mixture of 0·4 ml. of human serum, 0·4 ml. of 0·016 M NPS in 0·5 M acetate buffer, pH 7·5, and 2 ml. of N-NaOH. This curve is the sum of three factors: ○, the alkaline hydrolysis of NPS (water substituted for serum); ◓, the increase in the turbidity of the serum (water substituted for the NPS solution); ×, the serum-catalysed alkaline hydrolysis of NPS (curve ●, minus curves ○ and ◓).

the catalysed hydrolysis of *p*-nitrophenyl sulphate observed in the present work although the effect of proline and hydroxyproline, which were not examined by Perenyi, cannot be explained in this way.

Potassium *p*-nitrophenyl sulphate has been used freely in the past as a substrate for the assay of mammalian and other arylsulphatases (Dodgson *et al.* 1955; Dodgson, Melville, Spencer & Williams, 1954). It was not inconceivable that erroneous results might have been obtained in such assays owing to the failure to recognize any protein-catalysed alkaline hydrolysis of NPS which might have occurred during the period between addition of alkali at the end of the incubation period and the spectrophotometric measurement. However, when 10% (w/v) suspensions of rat liver, kidney, brain and intestine were substituted for serum in the assay procedure no release of *p*-nitrophenol from NPS after the addition of alkali could be detected. The concentration of protein used in this experiment was much greater than that used in previous assay work (Dodgson *et al.* 1955) and it can be assumed that the arylsulphatase activities measured in the previous studies are therefore correct. It is recommended, however, that NPS as an assay substrate should not be used under new conditions or for new sources of the enzyme until preliminary experiments have shown that non-enzymic liberation of *p*-nitrophenol under the alkaline conditions necessary for the formation of the *p*-nitrophenol anion does not occur.

RESULTS

Activity of serum and urine towards APS and NPS

The arylsulphatase *C* of human tissues is very active towards APS and NPS compared with aryl sulphatases *A* and *B*, which have little activity towards these substrates. The use of APS and NPS under the optimum conditions for human arylsulphatase *C* (0·005M APS, pH 6·9; 0·008M NPS, pH 7·5) provides a reasonably accurate measure of the activity of arylsulphatase *C*, since under these conditions the activity of, and competition by, arylsulphatases *A* and *B* are negligible (Dodgson *et al.* 1955, 1956). Assays of human sera and urines under these conditions showed that, apart from infected urines and those from adult females, none of the samples assayed had an activity greater than 0·24 NPS unit/ml. or 0·21 APS unit/ml. (Table 1). Three samples of sera from each of ox, sheep, dog, rabbit and rat were assayed with both substrates, but the activities found were no greater than those of human sera. The low level of arylsulphatase activity of urine and sera can be assessed by comparison with the APS activity of fresh human liver, which has varied between 4000 and 20000 units/g. wet wt. in the samples so far tested.

Apart from the higher activities found in the urine of adult females, the activities of normal urine and sera found with both substrates after a 20 hr. incubation period correspond to $E_t - E_t$ readings in the range 0–0·09. It is difficult to decide whether these small differences between test and control readings can be completely attributed to the activity of an arylsulphatase since the assay controls

Table 1. *Activity of urine and serum towards APS, NPS and NCS*

Urine and serum were assayed under the conditions described in the text. In some cases urines were centrifuged for 15 min. at 3000 *g* at the pH of assay and the cell-free supernatants assayed. The urines were not 24 hr. specimens. Ranges are quoted in parentheses. M, male; F, female.

Sample	Sex	No. of samples	Activity (μg. of phenol/hr./ml.) APS	NPS	NCS
Serum					
Normal	M	10	0·11 (0–0·21)	0·14 (0–0·21)	—
Normal	F	6	0·09 (0–0·19)	0·13 (0–0·23)	—
Normal	M	7	—	—	4·4 (2·8–7·8)
Normal	F	5	—	—	4·7 (2·2–6·5)
Two months before parturition	F	12	0·09 (0–0·18)	—	—
Urine					
Normal	M	26	0·06 (0–0·13)	—	—
Normal	M	12	—	0·12 (0·03–0·24)	—
	After centrifuging		—	0·10 (0·03–0·20)	—
Normal*	F	10	—	0·41 (0·09–0·82)	—
	After centrifuging		—	0·18 (0·07–0·26)	—
Normal	M	10	—	—	8·1 (6·3–11·9)
	After centrifuging		—	—	8·1 (6·3–11·9)
Normal	F	8	—	—	6·4 (3·4–10·3)
	After centrifuging		—	—	6·4 (3·4–10·3)
Infected	M	4	—	0·53 (0·27–1·06)	11·3 (7·8–17·6)
	After centrifuging		—	0·09 (0·01–0·16)	11·3 (7·8–17·6)

* The urine from one female had an NPS activity of 444, which was reduced to 32·4 on removing cellular debris by centrifuging. This urine was found to be contaminated with menses and has not been included in the table.

do not cover the possibility of a weak non-enzymic hydrolysis of the arylsulphate substrate by factors present in serum and urine. Separate experiments with boiled serum were designed to test this possibility but no satisfactory conclusion could be reached, since the coagulum formed by boiling the serum, even when diluted five times, would not dissolve in the assay incubation mixture. When assayed with either APS or NPS, boiled urines from males gave $E_t - E_c$ readings which were about one-third of those given by the corresponding untreated urines.

Arylsulphatase activity of urines from females. The urines from females consistently showed higher APS and NPS activities than those from males although there was no corresponding sex distinction between the enzyme activities of the sera (Table 1). Female urine generally contains large numbers of squamous epithelial cells derived mainly from the vagina and urethra. On the other hand, male urine normally contains very few epithelial cells, most of which are derived from the urinary tract. After removal of the cellular debris by centrifuging at 3000 *g* for 15 min. at the pH of assay, the NPS activity of the female urines fell from an average of 0·41 unit/ml. to an average of 0·18 unit/ml. (Table 1). The latter value is close to that found for untreated male urines (0·12 unit/ml.). There was no appreciable difference between the activities of centrifuged and untreated male urines. Four infected male urines containing large numbers of leucocytes showed high NPS activity, which could be removed by centrifuging (Table 1).

Effect of serum and urine on the activity of arylsulphatase C. It was possible that the arylsulphatase activities of urine and serum towards APS and NPS were being affected by endogenous inhibitors or activators. The low arylsulphatase activity of these fluids precluded the search for endogenous inhibitors by the method of Maengwyn-Davies & Friedenwald (1954), but it was possible to assess the effects of serum and urine on the arylsulphatase *C* of human liver in the following system: 0·2 ml. of APS (0·025 M) in 0·5 M acetate, pH 6·9; 0·4 ml. of acetone-dried human liver suspension in M acetate, pH 6·9 and 0·4 ml. of water, serum or urine adjusted to the assay pH. Serum had no effect on the activity of arylsulphatase *C* but urine inhibited strongly (30–60 %), the degree of inhibition varying with the sample. Assuming that the activity of urine towards NPS and APS is due to an enzyme similar to arylsulphatase *C* of liver, the true urinary activity, although possibly 50 % greater than the apparent value (Table 1), is still extremely low.

Activity of serum and urine towards NCS

Arylsulphatases *A* and *B* of human tissues are highly active towards NCS (Dodgson *et al.* 1956) and, since arylsulphatase *C* has only weak activity towards this substrate (Dodgson *et al.* 1956), NCS can be used to obtain an approximate measure of the combined activity of the two mitochondrial enzymes. Boyland *et al.* (1955) have already shown that serum and urine possess considerable NCS-hydrolysing activity and it has been possible to confirm many of their findings.

The optimum conditions for NCS-hydrolysing activity of urine were found to be 0·03 M NCS and pH 5·6 when measured over an incubation period of either 2 or 20 hr. Under these conditions a slight decrease in the rate of enzyme action occurred during the first 30 min. of incubation, but thereafter, up to 20 hr., a straight-line relationship existed between the nitrocatechol liberated and the time of incubation. The activities of male and female urines measured over a 2 hr. incubation period under the optimum conditions are shown in Table 1.

The substrate-concentration curve for serum acting on NCS was similar to that found for ox sulphatase *B* (Roy, 1954), the curve still ascending at 0·06 M NCS, above which concentration it is not practical to proceed owing to the insolubility of the substrate. In the presence of 0·04 M NCS the optimum pH for the arylsulphatase activity of serum towards NCS was in the region of 5·6. The activities of several different samples of serum under these conditions are recorded in Table 1.

Effect of urine on arylsulphatases A *and* B. Boyland *et al.* (1955) have shown that the arylsulphatase activity of urine towards NCS is markedly affected by endogenous inhibitors present in the urine. This was confirmed in the present work, there being a marked decline in the curve obtained by plotting enzyme activity (V) against enzyme concentration ($[E]$). Moreover, the plot of $[I]/V$ against $[I]$, where $[I]$, the inhibitor concentration, is proportional to $[E]$ (see Maengwyn-Davies & Friedenwald, 1954), was not a straight line, thus showing that the inhibition was not due to the effect of one inhibitor on a single enzyme. It has recently been shown that normal centrifuged urine contains two arylsulphatases which are active towards NCS and which possess electrophoretic mobilities identical with those of purified human-liver arylsulphatases *A* and *B* respectively (Dodgson & Spencer, 1956*b*). One of these enzymes, the electrophoretic mobility of which is identical with that of liver arylsulphatase *A*, when separated from urine, exhibits anomalous enzyme kinetics similar to those shown by the latter enzyme (Dodgson & Spencer, 1956*c*). It seems certain, therefore, that the NCS-hydrolysing activity of urine is due to the presence of both arylsulphatases *A* and *B*, and for this reason the effect of urine on the activity of purified liver arylsulphatases *A* and *B* was investigated (Table 2). It can be seen from this table

Table 2. *Effect of urine on arylsulphatases* A *and* B

Electrophoretically purified arylsulphatases *A* and *B* (Dodgson *et al.* 1956) were diluted with water, urine, boiled urine or urine which had been dialysed against running tap water for 24 hr. The mixtures were adjusted to the assay pH (5·0 for *A* and 6·0 for *B*) and 0·4 ml. was incubated with 0·4 ml. of NCS solution (0·01 M NCS in 0·5 M acetate, pH 5·0, for arylsulphatase *A* and 0·04 M NCS in 0·2 M acetate, pH 6·0, for arylsulphatase *B*) for 1 hr. at 38°. Alkaline quinol mixture (Roy, 1953), 2 ml., was added and the liberated 4-nitrocatechol measured at 515 mμ. Suitable controls were made to allow for the NCS-hydrolysing activity of the various urine preparations under these conditions.

	Arylsulphatase *A*		Arylsulphatase *B*	
	$\text{Log}_{10}(I_0/I)$	Inhibition of enzyme (%)	$\text{Log}_{10}(I_0/I)$	Inhibition of enzyme (%)
Purified enzyme	0·622	—	0·268	—
Urine	0·057	—	0·099	—
Boiled urine	0·016	—	0·003	—
Dialysed urine	0·041	—	0·055	—
Enzyme + urine	0·243	70·2	0·120	92·1
Enzyme + boiled urine	0·193	70·6	0·034	88·4
Enzyme + dialysed urine	0·638	3·6	0·318	1·9

that both enzymes retain activity in the presence of urine. Both enzymes therefore contribute towards the arylsulphatase activity of urine, although the greatest contribution would appear to be made by enzyme *A*. Although the NCS-hydrolysing activity of urine does not show the anomalous time–activity curves which are characteristic of purified liver arylsulphatase *A* (Dodgson & Spencer, 1956*c*) and does not show the same optimum conditions of pH and substrate concentration (Dodgson *et al.* 1956), these discrepancies can almost certainly be attributed to the presence of enzyme *B* in the urine and, more particularly, to the high concentrations in urine of inorganic phosphate and sulphate, both of which are known to modify considerably the kinetics of human-liver arylsulphatase *A* (Dodgson & Spencer, 1956*c* and unpublished results).

The urinary inhibitors of both liver arylsulphatases *A* and *B* were thermostable and dialysable (Table 2) and may be presumed to consist mainly of phosphate and sulphate ions, both of which, when present in concentrations similar to those obtaining in urine, are known to inhibit these enzymes (Dodgson *et al.* 1956 and unpublished results).

DISCUSSION

The presence in urine of arylsulphatases possessing similar properties and identical electrophoretic mobilities (see Dodgson & Spencer, 1956*b*) to those of arylsulphatases *A* and *B* of human liver accounts for the hydrolytic activity of urine towards nitrocatechol sulphate. Both these enzymes are known to occur in many human tissues (Dodgson *et al.* 1956) and although, for practical reasons, no close examination of serum has been attempted, it is probable that the same enzymes are responsible for the NCS-hydrolysing activity of serum. It is doubtful whether serum or urine contains an enzyme similar to the 'insoluble' or 'microsomal' arylsulphatase *C* of liver and other tissues (Dodgson *et al.* 1956), since both serum and urine show almost negligible activity towards NPS and APS under conditions of assay which are optimum for this enzyme. However, appreciable NPS-hydrolysing activity is occasionally observed in urine, but in these cases it can be attributed to the presence of cellular debris or bacteria and can therefore be removed from the urine by centrifuging.

Since the arylsulphatase activities obtained for urine and serum with NPS during the present work (0–0·8 μg. of *p*-nitrophenol liberated/hr./ml.) differed considerably from those obtained by Huggins & Smith (1947) (0·3–19·7 μg./hr./ml.) careful repetition of their work was undertaken. The procedure employed by Huggins & Smith differed from that of the present authors in four respects: the conditions used by the former, 0·001 M NPS at pH 5·8, were those established for the arylsulphatase of *Aspergillus oryzae*; an antibacterial agent, thymol, was incorporated into the incubation mixture; the volumes of substrate, buffer, serum or urine sample and NaOH were different; liberated *p*-nitrophenol was measured with a 420 mμ. filter. It was possible that these differences in experimental procedure might account for the conflicting results, but exact repetition of the method of Huggins & Smith (1947) by ourselves and several independent workers in this Laboratory have failed to disclose an activity in serum or urine greater than 0·14 μg./ml./hr. Four separate preparations of NPS gave similar results. In this connexion it is worth noting that Dr H. Cohen (personal communication) failed to detect arylsulphatase activity in horse serum by the method of Huggins & Smith (1947). The discrepancies in the observations of the two groups of workers are therefore still unexplained.

The results of any quantitative estimation of the arylsulphatase activities of serum and urine must be

interpreted with caution. When enzyme activity is measured with NCS, two arylsulphatases (*A* and *B*) compete for the same substrate. Moreover, in urine, natural substrates (the so-called ethereal sulphates) are also present. The activity of both arylsulphatases *A* and *B* is affected by certain inorganic ions, including sulphate, phosphate and chloride, and these are present in very variable amounts in urine. It is also possible that the pH optima of the two enzymes may vary slightly with the relative concentration of inorganic ions, as shown for arylsulphatases *A* and *B* of ox liver (Roy, 1955). The anomalous kinetics of arylsulphatase *A*, which are markedly affected by varying concentrations of inorganic ions (Dodgson & Spencer, 1956*c* and unpublished results), must also be borne in mind.

Although ureteric urine is known to possess arylsulphatase activity (Boyland *et al.* 1955) it is possible that some of the NCS-hydrolysing activity of urine is derived from the cells of the urinary and genital tracts. It is known that the NPS-hydrolysing activity of female urine which has been collected in a normal manner is largely due to the presence of vaginal epithelium cells, and the NPS-hydrolysing activity of infected urines is also due to sedimentable material, whether bacteria, leucocytes or erythrocytes. Such factors apparently contribute little to the NCS-hydrolysing activity of urine, since there is little difference in this activity between whole and centrifuged urines. However, it seems pertinent to point out that both arylsulphatases *A* and *B* are readily obtained in soluble form from liver cells by simple treatment such as incubation with various saline solutions, whereas the NPS-active arylsulphatase *C* is insoluble and remains attached to the particulate material in the cells (Dodgson *et al.* 1956). An approximate idea of the amount of NCS activity which could arise from the cellular debris of urine can be obtained by multiplying the difference between the NPS activity of urine and centrifuged urine by a factor (1·5), which represents the approximate average NPS:NCS ratio for human tissues [range, 1:0·39 (liver) to 1:4·1 (brain); Dodgson *et al.* 1956]. Thus the average NPS activity of female urine is 0·41 μg. of *p*-nitrophenol/hr./ml., and that of the centrifuged urines 0·18. The difference will correspond to an NCS activity of 0·34 μg. of 4-nitrocatechol/hr./ml., which is small when compared with the average NCS activity of female urines of 4·86. It is clear then, that in normal male and female urine only a small contribution is made to the NCS activity by the urinary sediment. Indeed, Boyland *et al.* (1955) found no correlation between NCS activity and red and white corpuscles and epithelial-cell counts in normal urine. However, in disease the urinary cellular debris may be substantial and it is suggested that in the interpretation of elevated arylsulphatase levels account should be taken, in the manner suggested above, of the difference in NPS activity between whole and centrifuged urine.

SUMMARY

1. Human serum and urine exhibit little arylsulphatase activity towards potassium *p*-acetylphenyl and *p*-nitrophenyl sulphates. In certain circumstances urine does show activity towards these substrates but the activity resides in sedimentable material such as cellular debris and bacteria.

2. Both serum and urine exhibit appreciable arylsulphatase activity towards nitrocatechol sulphate. The activity in urine is a feature of the urine *per se* and probably arises only in part from cellular debris.

3. Two enzymes, corresponding to arylsulphatases *A* and *B* of human tissues, are responsible for the bulk of the arylsulphatase activity of urine.

4. Some observations on the non-enzymic catalysis by serum of the alkaline hydrolysis of *p*-nitrophenyl sulphate are reported.

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The Kinetics of Hydrolysis of Phenyl Phosphate by Alkaline Phosphatases

By R. K. MORTON
*Department of Biochemistry, University of Cambridge**

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Alkaline phosphatases purified from cow's milk and calf intestinal mucosa have a similar substrate specificity. However, under optimum conditions, the rate of hydrolysis of typical substrates by intestinal phosphatase is about five times that of the milk enzyme (Morton, 1955*a*). A comparative study of the kinetic behaviour of the two enzymes therefore appeared desirable. Variation of such properties as maximum initial velocity of hydrolysis ($V_{max.}$) and Michaelis constant (K_m) with pH might be expected to indicate whether the same or a different mechanism was involved in the hydrolysis of substrate by these two enzymes. Moreover, determination of the kinetics of substrate hydrolysis was an essential preliminary to investigation of the kinetics of the transfer reactions catalysed by phosphatases (Morton, 1952, 1953*a*).

Milk phosphatase is derived from mammary-gland microsomes which are secreted with milk (Morton, 1953*b*, 1954*a*). This and other evidence has established that the milk and mammary-gland alkaline phosphatases are identical (Morton, 1955*a*). By using a crude preparation of alkaline phosphatase from guinea-pig mammary gland, Folley & Kay (1935) found that the optimum pH for hydrolysis of monophenyl phosphate varied with substrate concentration. Preliminary findings with purified cow's milk phosphatase were in reasonable agreement with those of Folley & Kay (1935). Hydrolysis of monophenyl phosphate by purified calf intestinal phosphatase was investigated in detail, extending the range of conditions used by Folley & Kay (1935) with mammary-gland (milk) enzyme. The results are described in this paper. This work was carried out in 1950 and, although not previously published, has been described elsewhere (Morton, 1952).

MATERIALS

Monophenyl phosphate. A commercial preparation of the disodium salt (British Drug Houses Ltd.) was dissolved in water, free phenol removed by extraction with diethyl ether, and inorganic phosphate precipitated with ammoniacal magnesia. The product was dried *in vacuo* over conc. H_2SO_4 at room temp.

For metal-activation studies, this product was dissolved in a minimum of hot glass-distilled water, and metal-free 6N-HCl added until the pH was about 2 (at 20°). The solution was cooled to about 2° and excess of redistilled ethanol added to precipitate about half the phenyl phosphate acid, which was washed with ethanol and with diethyl ether and dried *in vacuo* over conc. H_2SO_4 at room temp. The product was dissolved in redistilled ethanolamine (4M) to give a concentrated solution at the required pH value.

Buffers. These were purified and prepared as described previously (Morton, 1955*a*). Veronal (sodium diethylbarbiturate)–sodium acetate–HCl buffers (Michaelis, 1931) were used for pH values between 7 and 9, and ethanolamine–HCl buffers for values greater than 9. For metal-activation studies, twice-recrystallized diethylbarbituric acid was dissolved in ethanolamine so that the resultant solution was 0·25M with respect to both components. This veronal–ethanolamine buffer was adjusted to the required pH with either metal-free 6N-HCl or 2N-NaOH.

Enzymes. Alkaline phosphatases purified from calf intestinal mucosa (Morton, 1950, 1954*b*) and cow's milk (Morton, 1950, 1953*c*) were used throughout.

METHODS

pH *values.* Except where otherwise indicated, all values given refer to pH at 38°. They were determined with replicate tubes of the buffered substrates used for activity measurements.

Inorganic phosphate. This was estimated by the method of Fiske & Subbarow (1925) or, in metal-activation studies, by the method of Horwitt (1952).

Phenol. This was estimated by the method of King (1951), modified as previously described (Morton, 1955*b*).

Initial rate of hydrolysis (V_0). For the kinetic studies, this was determined by estimating both the inorganic phosphate and the phenol liberated from phenyl phosphate in 5 min. at 38°. There was generally good agreement between the two determinations, but values obtained with the more sensitive phenol method were preferred where very low substrate concentrations were used. The procedure was essentially as described previously (Morton, 1953*d*). The reaction was commenced by addition of 0·1 ml. of enzyme solution to 4·9 ml. of buffered substrate, and stopped by the addition of 0·2 ml. of 20% (w/v) trichloroacetic acid. Results are expressed as μg. of P liberated in 5 min. by 0·1 ml. of the standard solution of enzyme. This contained in 1 ml. approx. 55 enzyme units, as previously defined (Morton, 1953*d*), equivalent to approx. 4·1 μg. of purified intestinal phosphatase or to approx. 22 μg. of purified milk phosphatase.

* Present address: Department of Biochemistry, University of Melbourne.

It was confirmed that the initial velocity of hydrolysis was linear up to 10% hydrolysis of the substrate (Morton, 1953*d*). Except at the lowest substrate concentration ($2{\cdot}5 \times 10^{-5}$ M), the hydrolysis in no case exceeded 10%.

RESULTS

Phosphatase from calf intestinal mucosa

Influence of substrate concentration on the activity–pH curve. The initial velocity of hydrolysis of phenyl phosphate was determined at several pH values over the range 8·25–10·1 and at eight substrate concentrations from $2{\cdot}5 \times 10^{-5}$ M to $7{\cdot}5 \times 10^{-2}$ M. The results are shown in Fig. 1.

At low substrate concentration (Fig. 1, curves *A–C*), the optimum pH is not well defined. An observation at pH 7·6 with $2{\cdot}5 \times 10^{-5}$ M substrate showed little further change of V_0 from that shown for pH 8·2 (Fig. 1, curve *A*), so that V_0 is fairly constant over the pH range 7·6–9·0 at this very low substrate concentration. However, hydrolysis is very slow at pH values above 9·6 (Fig. 1, curve *A*). This has an important bearing on the measurement of phosphotransferase activity (Morton, 1952, 1953*a*). Observations made since this experiment was carried out show that, for the highest substrate concentration ($7{\cdot}5 \times 10^{-2}$ M; curve *H*), the optimum pH is about 10·0 and the activity at this pH is slightly greater than the maximum shown for curve *G*.

Fig. 1 shows that the optimum pH for hydrolysis varies with the substrate concentration and the optimum substrate concentration also varies with the pH. Fig. 2 shows that the relationship between the optimum pH for hydrolysis (as estimated from Fig. 1) and p[*S*] is linear over the substrate range $2{\cdot}5 \times 10^{-5}$ to $2{\cdot}5 \times 10^{-3}$ M, but deviates considerably from linearity at higher substrate concentrations. There is some uncertainty about the optimum pH values at very low substrate concentrations, since these are not well defined (Fig. 1).

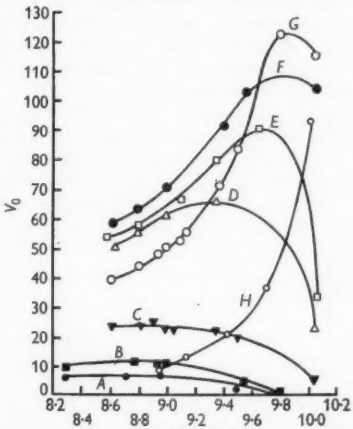

Fig. 1. Plot of initial rate of hydrolysis, V_0, against pH for hydrolysis of phenyl phosphate at various concentrations. The reaction mixture (5 ml.) contained either veronal–sodium acetate–HCl buffer (0·05 M), for pH values less than 9, or ethanolamine–HCl buffer (0·05 M) for pH values greater than 9, magnesium acetate (0·01 M), and approx. 5·5 units of purified calf intestinal alkaline phosphatase. The reaction was carried out for 5 min. at 38°, and enzymically liberated phenol and inorganic phosphate were estimated as described in the text. The curves are for the following substrate concentrations (M): *A*, $2{\cdot}5 \times 10^{-5}$; *B*, 5×10^{-5}; *C*, 10^{-4}; *D*, 5×10^{-4}; *E*, $7{\cdot}5 \times 10^{-4}$; *F*, $2{\cdot}5 \times 10^{-3}$; *G*, $2{\cdot}5 \times 10^{-2}$; *H*, $7{\cdot}5 \times 10^{-2}$.

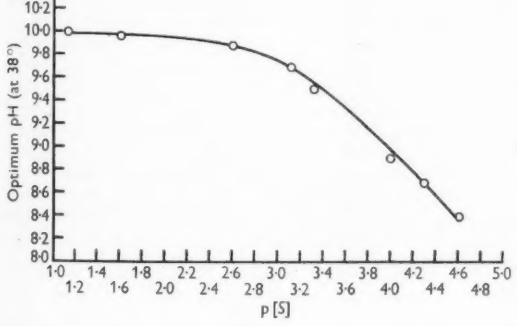

Fig. 2

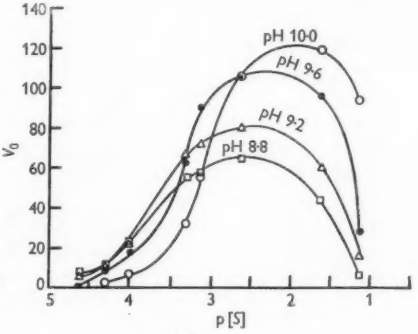

Fig. 3

Fig. 2. Dependence of optimum pH for hydrolysis of phenyl phosphate by alkaline phosphatase on substrate concentration. Values of optimum pH were obtained from Fig. 1.

Fig. 3. Influence of phenyl phosphate concentration on the initial rate of hydrolysis by intestinal alkaline phosphatase at several pH values. Values of V_0 were obtained from Fig. 1.

Values of V_0 for various substrate concentrations at regular pH increments of 0·2 unit from pH 8·6 to 10·0 were obtained from Fig. 1. Plots of V_0 against p[S] (Michaelis & Menten, 1913) for pH values 8·8, 9·2, 9·6 and 10·0 are shown in Fig. 3. The inhibition by high substrate concentrations is apparent at all pH values. Especially noteworthy is the relatively narrow range of substrate concentrations over which optimum activity is obtained.

Variation of K_m with pH. Plots of $1/V_0$ against $1/[S]$ (Lineweaver & Burk, 1934) are shown in Fig. 4 for pH values 9·0, 9·4 and 9·8. The non-linear relationship is apparent. This is even more pronounced if the results for the lowest substrate concentration ($2{\cdot}5 \times 10^{-5}$ M) are included. They were omitted from Fig. 4 for the sake of clarity, as were the curves for other pH values investigated.

Values for K_m were estimated from Michaelis–Menten plots, as the substrate concentration for one-half maximum velocity. The Dixon (1953) plot of pK_m against pH is shown in Fig. 5 (curve A). This reveals a -1 unit slope at higher pH values, changing to zero slope at lower values, the discontinuity appearing at about pH 9·2.

A similar plot for mammary gland (milk) alkaline phosphatase, based on the data of Folley & Kay (1935), is shown in Fig. 5 as curve B. Although the data are limited, here also there are indications of a -1 unit slope at higher pH values, with a discontinuity at about pH 9·2. Some values obtained with purified milk phosphatase are included in curve B in Fig. 5.

Turnover number of intestinal alkaline phosphatase. As shown by Fig. 4, the Lineweaver–Burk plots are non-linear at several pH values, especially when considered over a wide substrate range. Plots of $[S]/V_0$ against $[S]$, and of V_0 against $V_0/[S]$ (Hofstee, 1952), were also found to be non-linear, as expected. Hence it is difficult to obtain any reliable estimate of the variation with pH of $V_{max.}$, the maximum rate for infinite substrate concentration. However, from the plot of V_0 against pH (Fig. 1), a maximum observed velocity ($U_{max.}$) was obtained for each substrate concentration. Such values, of course, are obtained at different pH values according to the substrate concentration. A plot of $1/U_{max.}$ against $1/[S]$ is shown in Fig. 6. The plot is quite linear and gives a value for V_{max} of 250 μg. of P/5 min./0·1 ml. of enzyme. On this basis, the turnover number of purified calf intestinal phosphatase is about

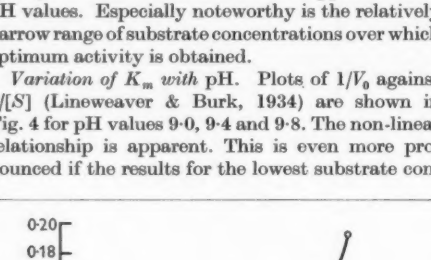

Fig. 4. Lineweaver–Burk plots (reciprocal of the initial rate of hydrolysis against the reciprocal of the substrate concentration) for intestinal alkaline phosphatase with phenyl phosphate at various pH values. Values of V_0 were obtained from Fig. 1.

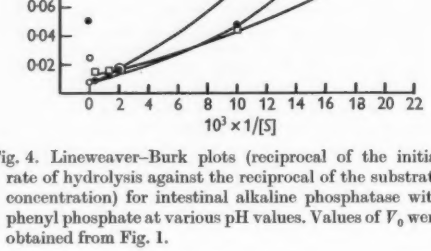

Fig. 5. Dixon plots (pK_m against pH) for hydrolysis of phenyl phosphate by alkaline phosphatases. Curve A is for intestinal phosphatase and curve B for milk phosphatase. Curve B includes results of Folley & Kay (1935) marked thus: ●.

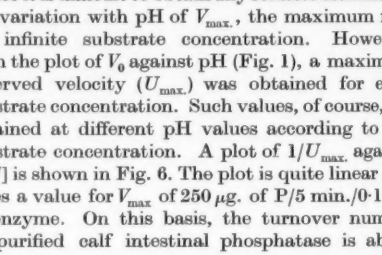

Fig. 6. Lineweaver–Burk plot (reciprocal of $U_{max.}$ against reciprocal of substrate concn.), where $U_{max.}$ is the maximum observed rate of hydrolysis of phenyl phosphate at each substrate concn. The values of $U_{max.}$ are for intestinal alkaline phosphatase as determined from Fig. 1.

394 000 moles of phenyl phosphate hydrolysed/min./100 000 g. of protein at 38°. Taking the molecular weight as 60 000 (Schramm & Armbruster, 1954), the turnover number is 236 000 moles/min./mole of enzyme at 38°.

Influence of metal salts on phosphatase activity. As shown previously (Morton, 1955*a*), purified intestinal phosphatase is activated by magnesium and manganese salts, but nevertheless shows considerable activity in the absence of such salts, even after extensive dialysis against water. Some aspects of metal activation were re-investigated with intestinal phosphatase which had been purified to stage 6 (Morton, 1954*b*, Table 4) and held in the dried state over $CaCl_2$ at room temp. for about 4 years. After dialysis of solutions of this preparation in Visking tubing against glass-distilled water, the behaviour in response to added $MgCl_2$ and $MnCl_2$ was generally similar to that already described (Morton, 1955*a*).

The enzyme was then treated with a metal-chelating agent in an attempt to remove metal firmly bound to the enzyme. About 10 mg. of the phosphatase was dissolved in 10 ml. of 0·1 M disodium ethylene diaminetetraacetate (EDTA), previously brought to pH 8·5 with M ethanolamine. The solution was dialysed in Visking tubing against 0·1 M-EDTA at pH 8·5, and subsequently against frequent changes of glass-distilled water for 6–16 hr. at 2–4°.

This treatment caused considerable loss of activity, which was partly restored by addition of $MgCl_2$ (5×10^{-3} M) and, to a lesser extent, by $MnCl_2$ (10^{-3} M). As shown in Table 1, the extent of activation by $MgCl_2$ depends on the concentration of substrate, the activation being relatively greater for low concentrations of phenyl phosphate. From Table 1 it is seen that the relationship between the reciprocal of V_0 and the reciprocal of the concentration of added metal salt ($[M]$) is grossly non-linear at both concentrations of substrate.

Table 1. *Influence of phenyl phosphate concentration on the activation of intestinal alkaline phosphatase by magnesium chloride*

The reaction mixture (5 ml.) contained veronal–ethanolamine buffer (0·05 M with respect to both components) at pH 9·6, $MgCl_2$ and phenyl phosphate at the concentrations indicated, and about 1·5 units of enzyme. The reaction was for 10 min. at 38°. Values of $MgCl_2$, $[M]$, are expressed as molar concentrations, and the initial rates of hydrolysis, V_0, as μg. of P liberated/10 min./0·2 ml. of enzyme solution. The results are given as reciprocals.

	$10^4/V_0$	
$1/[M]$	At $2{\cdot}5 \times 10^{-3}$ M phenyl phosphate	At $2{\cdot}5 \times 10^{-4}$ M phenyl phosphate
10^8	228	2500
10^7	193	1620
10^6	152	1600
10^5	135	1430
10^4	130	435
10^3	128	322
10^2	115	303

Fig. 7 shows that the optimum pH for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-3}$ M) is more than one higher with $MgCl_2$ (5×10^{-3} M) than with $MnCl_2$ (10^{-3} M). There was no substantial difference in the pH optima obtained with high (10^{-2} M) and low (10^{-5} M) concentrations of $MgCl_2$, although the activity was much reduced at the lower concentration. Essentially the same opimum pH for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-2}$ M) was obtained with large amounts of enzyme in the absence of added $MgCl_2$ as with about one-twentieth of the enzyme with $MgCl_2$ (10^{-2} M).

Preliminary observations with phosphatase from cow's milk

The activity–pH curve for hydrolysis of phenyl phosphate ($2{\cdot}5 \times 10^{-3}$ M) by the milk phosphatase showed an optimum pH of 10·05 (Morton, 1955*b*). The shape of the curve for V_0 against pH was generally similar to that obtained by Folley & Kay (1935), who obtained an optimum pH of 10·05 for

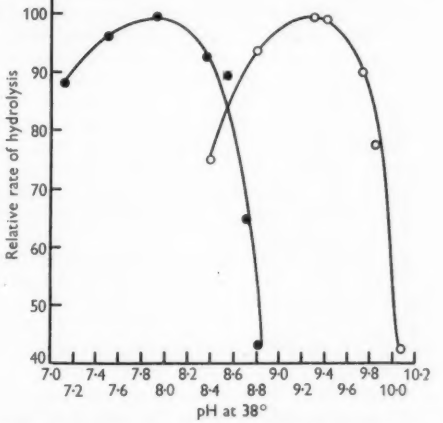

Fig. 7. Dependence of the optimum pH for hydrolysis of phenyl phosphate by intestinal alkaline phosphatase on the nature of the activating cation. The reaction mixture (2 ml.) contained phenyl phosphate ($2{\cdot}5 \times 10^{-4}$ M), veronal–ethanolamine buffer (0·05 M with respect to both components), and either $MgCl_2$ (0·005 M) with about 0·1 unit of enzyme (○), or $MnCl_2$ (0·001 M) with about 0·6 unit of enzyme (●). The reaction was for 10 min. at 38°. The results are expressed as relative rates of hydrolysis, the maximum value in each case being assigned a value of 100.

hydrolysis of phenyl phosphate ($4{\cdot}5 \times 10^{-3}$ M) by an extract of guinea-pig mammary gland. Moreover, as shown in Fig. 5 (curve *B*), the K_m values at pH 9·6 and 10·0 were also in close agreement with values obtained at or near the same pH by Folley & Kay (1935).

No further kinetic work was carried out with the milk phosphatase, but the observations suggest that the results of Folley & Kay (1935) may be tentatively accepted as applying to cow's milk phosphatase.

DISCUSSION

The marked dependence of the optimum pH for hydrolysis upon substrate concentration (Figs. 1, 2) appears to be characteristic of alkaline phosphatases from kidney (Asakawa, 1928; Jacobsen, 1932, 1933) and mammary gland (Folley & Kay, 1935) as well as from intestine. Moreover, with the intestinal enzyme this dependence is found with substrates other than phenyl phosphate, as is shown by the results of Ross, Ely & Archer (1951), which appeared sometime after the work described here had been completed (see Morton, 1952). Especially noteworthy is the broad pH optimum (extending towards pH 7) obtained with low concentrations of phenyl phosphate (Fig. 1). The optimum pH values obtained with phenyl phosphate are considerably higher than with β-glycerophosphate and most naturally occurring organic phosphates (Delory & King, 1943; Morton, 1955*b*). These observations and those of Ross *et al.* (1951) support Neumann's (1949) suggestion that alkaline phosphatases may be quite active at so-called 'physiological' pH values (assumed to be about pH 7). If intracellular pH values may be varied (as, for example, by endocrine control), this would offer an effective means for controlling alkaline phosphatase activity and possibly, therefore, the intracellular concentration of phosphate esters.

The optimum pH values shown in Fig. 1 are probably due to reversible effects and not due to irreversible enzyme denaturation, since the incubation period was short (5 min.) and since the phosphatase is protected against alkaline pH effects by its substrate (Folley & Kay, 1935; Morton, unpublished work). Other workers, such as Ross *et al.* (1951), have employed incubation periods of up to 1 hr. The values so obtained are frequently not good estimates of the true initial rates, and the pH optima are appreciably lower than the true values owing to irreversible enzyme denaturation, as has been demonstrated by Folley & Kay (1935).

Fig. 2 shows that with phenyl phosphate as substrate, the optimum pH increases by unity for unit decrease of p[*S*] over the range 4·6–3·3, whereas there is very little change over the p[*S*] range from 3 to 1·1. The discontinuity between the two parts of the curve occurs at a p[*S*] value of about 3, and the limiting optimum pH is approached at p[*S*] values of less than 3. A similar plot (not shown here) of the results of Ross *et al.* (1951) with crude rat intestinal alkaline phosphatase and with β-glycerophosphate as substrate shows a linear relation between optimum pH and p[*S*] over the range 4·9–1·3, with a change of about pH 0·5 for unit change of p[*S*]. It is possible that studies of the change of optimum pH with p[*S*] for several different substrates could provide additional information concerning the mechanism of action of alkaline phosphatase.

From Figs. 1 and 2, it is clear that the rate of hydrolysis of substrate will rapidly decline if carried out at a fixed pH value such as that which gives the maximal initial rate. Where complete hydrolysis of a substrate is desired therefore the reaction should be initiated at a pH value considerably below the optimum value for the initial substrate concentration. Not only will the optimum pH for substrate cleavage change during the course of hydrolysis, but inhibition due to liberated inorganic phosphate will also change. This inhibition varies with pH (Jacobsen, 1933). It is therefore apparent that time curves for hydrolysis of a substrate by alkaline phosphatase are quite complex and have limited value for kinetic purposes. This investigation has therefore been restricted to measurements of initial reaction rates.

Fig. 7 shows that the optimum pH for hydrolysis is dependent on the nature of the metal activator. Since the optimum pH obtained in the absence of added activator is substantially the same as with added magnesium chloride, it seems probable that the activity in the absence of activator is due to the retention by the enzyme of a small amount of magnesium, even after treatment with EDTA. This supports the view expressed previously (Morton, 1955*a*) that purified intestinal phosphatase binds magnesium so strongly that it is not removed by extensive dialysis against water.

Complex kinetic behaviour of alkaline phosphatases

In considering the interpretation of (*a*) change of optimum pH with substrate concentration and change of optimum substrate concentration with pH (Figs. 1, 2); (*b*) inhibition at high substrate concentrations (Fig. 3) (the inhibition changing with pH); and (*c*) metal activation, which varies with the concentration of the substrate, it is necessary to take account of the various equilibria likely to influence the rate of formation of a complex and its decomposition to products, according to the classical formulation

$$E + S \rightleftharpoons ES \rightarrow E + P, \qquad (1)$$

in which *E* represents enzyme, *S* substrate and *P* the products of the reaction.

It is assumed that phosphatase activity is

dependent on the presence of a metal (M), especially magnesium (see Morton, 1955*a* and Table 1). Thus in a reaction mixture containing E, S and M, and which yields P, the following equilibria must be considered:

$$E+M \rightleftharpoons EM, \quad (2)$$
$$EM+S \rightleftharpoons EMS, \quad (3)$$
$$M+S \rightleftharpoons MS, \quad (4)$$
$$E+MS \rightleftharpoons EMS, \quad (5)$$
$$E+S \rightleftharpoons ES, \quad (6)$$
$$ES+M \rightleftharpoons EMS, \quad (7)$$
$$EMS+S \rightleftharpoons EMS_2, \quad (8)$$
$$M+(OH)_2 \rightleftharpoons M(OH)_2, \quad (9)$$
$$EMS \rightarrow EM+P, \quad (10)$$
$$EMS+M \rightleftharpoons EM_2S. \quad (11)$$

EMS is considered to be the only complex which undergoes decomposition to form the products of the reaction (equation 8).

It is clear that equations 3, 5 and 7 represent competitive routes for formation of EMS. Under these conditions, plots of $1/V_0$ against $1/[S]$, and of $1/V_0$ against $1/[M]$ will both be non-linear (see Friedenwald & Maengwyn-Davies, 1954, p. 154). The non-linear plots obtained (Fig. 4 and Table 1) suggest that such competitive pathways are operative. Although this kinetic evidence supports the existence of a complex between metal and substrate (equation 4), this has not been directly demonstrated. However, it has been observed that a precipitate of magnesium hydroxide from magnesium chloride (10^{-2}M) appears at a higher pH value when phenyl phosphate ($2{\cdot}5 \times 10^{-3}$M) is also present. Moreover, by measurement of paramagnetic-resonance absorption, Cohn (1954) has directly demonstrated complexes between phosphomonoesters (such as glucose 6-phosphate) and manganese.

Equation 9 shows that the concentration of hydroxyl ion will influence the concentration of effective activator, quite apart from the effect on the enzyme protein. If a complex is formed between S and M, then high substrate concentrations will, to some extent, counter the effect of high hydroxyl ion concentration. Thus equations 4 and 9 are competitive. At similar buffered substrate concentrations, the precipitate of manganese hydroxide appears at much lower pH values than does that of magnesium. This is in accordance with the known affinities of these two metals for hydroxyl ions (see Klotz, 1954). Hence the removal of activator as metal hydroxide (equation 9) will occur at lower pH values with manganese than with magnesium chloride. This would quite well account for the lowering of the pH optimum as well as for the considerably reduced activity when manganese is the activator as compared with magnesium salt (Fig. 7).

The curves shown in Fig. 3 reveal inhibition by excess of substrate. Haldane (1930) suggested that such inhibition may be due to the formation of an inactive complex of enzyme with two molecules of substrate (equation 8). For such a case, Haldane showed that

$$V_0 = \frac{k}{(1+K_1/[S]+[S]/K_2)},$$

where V_0 is the observed rate; $[S]$ is the substrate concentration; k is a constant; and K_1 and K_2 are the equilibrium constants for the reactions represented by equations 3 and 8 respectively. As discussed by Folley & Kay (1935), bell-shaped curves of the type shown in Fig. 3 are consistent with Haldane's formulation.

Although it is necessary to consider at least three routes to the formation of EMS (equations 3, 5 and 7), the same group of the enzyme is always involved in the binding of the substrate molecule in this active complex. It is reasonable to assume that a different group will be involved in the binding of a second substrate molecule to form the inactive complex EMS_2 (equation 8). Qualitatively it would be expected that, if the pK of the group involved in binding the first substrate molecule (in EMS) differs appropriately from the pK of the group binding the second molecule (in EMS_2), then there will be inhibition at high substrate concentrations, the extent of which will depend on pH. Moreover, the optimum pH will be expected to change with substrate concentration and the optimum substrate concentration with pH.

Friedenwald & Maengwyn-Davies (1954, p. 191) have shown that change of optimum pH with substrate concentration, together with change of optimum substrate concentration with pH, will be obtained if the substrate exists in two forms, one of which combines with the enzyme to form an active intermediate, the other of which acts as a non-competitive inhibitor. These workers derived an equation representing the relationship between V_0 and $V_{max.}$ under these conditions. They suggested that the results of Ross *et al.* (1951) for hydrolysis of β-glycerophosphate by rat intestinal phosphatase approximately fit such an equation, if the true substrate is S^- and the non-competitive inhibitor SH. The dissociation of SH must be substantially affected in the pH range under consideration. This is unlikely in the studies of Ross *et al.* (1951), which include the range pH 7·5–9·5, with β-glycerophosphate, pK 6·34 (Delory & King, 1943), as substrate; it is excluded in the present study over the pH range 8·25–10·0 with phenyl phosphate, pK 5·73 (Delory & King, 1943), as substrate.

Since with increasing concentrations of magnesium and manganese salts, the activity of purified phosphatase passes through a maximum, followed by a slight decline in activity (see Morton, 1955*a*,

Fig. 6), it is possible that more than one molecule of metal may be bound by the enzyme to form an inactive complex (EM_2S). This situation is represented by equation 11.

The results indicate that, over the range of substrate concentrations and pH conditions studied, all of the several equilibria represented by equations 2–11 may influence the rate of hydrolysis of phenyl phosphate by alkaline phosphatase. A general discussion of the kinetic situation where several equilibria are involved in enzymic catalysis has been given by Bull (1954), who has derived an equation expressing the rate of formation of the reaction products in terms of the concentrations of free enzyme and of the various enzyme complexes. Bull (1954) has applied this treatment to hydrolysis of urea by urease. The kinetic behaviour of alkaline phosphatase is rather similar to that of urease, as has been discussed elsewhere (Morton, 1952). However, whereas the optimum pH for alkaline phosphatase increases with increasing substrate concentration (Fig. 2), that for urease decreases (Van Slyke & Cullen, 1914; Van Slyke, 1942).

Interpretation of the Dixon plot for alkaline phosphatases

The -1 unit slope shown in the Dixon (1953) plot (Fig. 5, curve *A*), for pH values above 9·4, is found not only for intestinal and milk phosphatase with phenyl phosphate as substrate, but also for rat-kidney phosphatase with glycerophosphate as substrate (see Dixon, 1953). A similar plot (not shown here) of results of Roche & Sarles (1954) also gives approximately a -1 unit slope at pH values above 9 for dog intestinal, kidney and liver alkaline phosphatases with glycerophosphate as substrate. The results of Roche & Sarles (1954) for dog intestinal phosphatase also show a discontinuity at about pH 9·0, thus indicating that the discontinuity shown in Fig. 5 is probably independent of the nature of the substrate.

Interpretation of these results is dependent on the meaning of the experimentally determined K_m values. In view of the importance of the various equilibria previously discussed, it is clear that the K_m value is not identical with K_s, the dissociation constant of the enzyme–substrate complex. However, this need not necessarily invalidate the use of the Dixon plot (see Dixon, 1953). In view of the whole-number slopes (zero and -1) obtained (Fig. 5), it seems certain that the dissociation of a rate-determining enzyme complex is substantially affected in the pH range under consideration.

According to 'rule 9' of Dixon (1953), the -1 unit slope (Fig. 5, curve *A*) thus indicates that, at pH values above about 9·4, 'desubstration' of the rate-determining complex proceeds with gain of unit negative charge or with loss of unit positive charge, whereas there is no change of charge at pH values below 9·0. According to 'rule 10' of Dixon (1953), the discontinuity (Fig. 5, curve *A*) indicates the pK value of a group, either of the substrate or of the enzyme, which is involved in the reversible formation of the rate-determining complex. Since the pK value of the relevant group of phenyl phosphate is 5·73 (Delory & King, 1943), the discontinuity at about pH 9·2 must be the pK value either of an amino acid residue of the enzyme or of the hydroxide of the bound metal. Thus considering only amino acid residues of the enzyme, and neglecting the metal component and the ionized state of the substrate, the reversible formation of the active enzyme complex could be represented as involving either (*a*) a cationic or (*b*) an anionic group, as follows:

$$\left.\begin{array}{ll} & (a)\ E\mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S]^+ \\ \text{or} & (b)\ E\mathrm{H} + S \rightleftharpoons [E\mathrm{H}S] \end{array}\right\} \quad \text{below pH 9·0,}$$

and

$$\left.\begin{array}{ll} & (a)\ E + \mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S]^+ \\ \text{or} & (b)\ E^- + \mathrm{H}^+ + S \rightleftharpoons [E\mathrm{H}S] \end{array}\right\} \quad \text{above pH 9·4.}$$

The pK values of the ionizable groups of amino acids, when free and in some simple peptides, are fairly well established (see Cohn & Edsall, 1943, pp. 84 and 445). However, values listed by Steinhardt & Zaiser (1955) show that in proteins the pK value for any amino acid group may be shifted quite considerably, owing to interaction with neighbouring groups. Since either a cationic or an anionic group may be involved, as suggested by Dixon (1953), it is possible that the phenolic hydroxyl of tyrosine, the ϵ-amino of lysine, or the thiol group of cysteine may be the relevant group.

Since alkaline phosphatase is not inhibited by compounds which react more or less specifically with thiol groups (Barron & Singer, 1945; Roche, 1950), it is unlikely that the group is that of cysteine. The irreversible inhibition by keten (Gould, 1944) and the reversible inhibition by iodine in potassium iodide (Sizer, 1942; Morton, 1955*b*) indicate that the phenolic hydroxyl of a tyrosine residue is essential for enzymic activity, as first suggested by Sizer (1942). Hence the discontinuity at pH 9·2 probably represents the pK of a phenolic hydroxyl of a tyrosine residue, although the possibility that it is due to the ϵ-amino group of lysine (Morton, 1952) cannot be eliminated. Further identification of the relevant group could possibly be obtained by investigation of the apparent heat of ionization, since the values for the phenolic hydroxyl of tyrosine and for the ϵ-amino group of lysine are characteristically very different (Cohn & Edsall, 1943, p. 89; Alberty, 1953).

From Fig. 6 a K_m value of $9{\cdot}6 \times 10^{-4}$ M is obtained for calf intestinal phosphatase with phenyl phos-

phate. From a similar type of plot, Ross *et al.* (1951) obtained a value of 3×10^{-2}M for rat intestinal phosphatase with β-glycerophosphate. Figures obtained in this way could be most useful for expressing the affinity of alkaline phosphatase for different substrates. Since K_m values should always be expressed for a given pH, it is suggested that a figure obtained from a plot of $1/U_{max.}$ against $1/[S]$ (as in Fig. 3) should be termed the 'affinity index'. Such indices could be used to compare the affinities of alkaline phosphatase for different substrates. Indices obtained with the one substrate would be useful for comparing alkaline phosphatases of different origins.

Although the kinetic results for milk alkaline phosphatase are limited, the observations presented here and previously (Morton, 1955*a*), taken together with the classical studies of Folley & Kay (1935), suggest that the kinetic behaviour of milk phosphatase is not substantially different from that of intestinal phosphatase. Fig. 5, curve *B*, indicates a discontinuity in the Dixon plot at about pH 9·2. A similar interpretation to that already offered for intestinal phosphatase leads to the possibility that a tyrosine residue is concerned in the formation of a rate-limiting enzyme complex. The differences between intestinal and milk phosphatase, which have been outlined in a previous paper (Morton, 1955*a*), appear to reflect some difference in the enzyme–metal interaction.

SUMMARY

1. The hydrolysis of phenyl phosphate by purified alkaline phosphatase from calf intestinal mucosa has been investigated at substrate concentrations from $2{\cdot}5 \times 10^{-5}$M to $7{\cdot}5 \times 10^{-2}$M and at pH values from 8·25 to 10·1, the concentration of added magnesium chloride (enzyme activator) being maintained constant at 10^{-2}M.

2. There is marked inhibition by high concentrations of substrate, especially at lower pH values. The optimum pH for hydrolysis varies considerably with substrate concentration. The optimum substrate concentration also varies with pH. At a fixed substrate concentration, the optimum pH is influenced by the nature of the metal activator.

3. The existence of several equilibria involving enzyme, substrate and metal are postulated to explain these results. Evidence is presented which supports this hypothesis.

4. The Dixon (1953) plot of pK_m against pH reveals a discontinuity at about pH 9·2 (at 38°). This is interpreted as indicating that a phenolic hydroxyl group of a tyrosine residue of the enzyme is directly involved in the formation of a rate-limiting enzyme complex.

5. The turnover number of intestinal alkaline phosphatase is calculated to be about 394 000 moles of phenyl phosphate/min./100 000 g. of protein (236 000 moles/min./mole of enzyme) at 38°.

6. Results obtained with purified alkaline phosphatase from milk support earlier observations by Folley & Kay (1935) using alkaline phosphatase from mammary gland of the guinea pig. The behaviour of the milk (mammary-gland) phosphatase suggests that the several equilibria recognized for the intestinal phosphatase system are also important for the milk-phosphatase system.

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Fungal Detoxication

2. THE METABOLISM OF SOME PHENOXY-*n*-ALKYLCARBOXYLIC ACIDS BY *ASPERGILLUS NIGER**

By R. J. W. Byrde and D. Woodcock
Long Ashton Research Station, Bristol University

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The earlier paper in this series (Byrde, Harris & Woodcock, 1956) described the metabolism of ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids by *Aspergillus niger* van Tiegh. By using a replacement culture technique it was found that 2-naphthyloxyacetic and β-(2-naphthyloxy)propionic acids were hydroxylated in the 6-position; higher members of the homologous series underwent both nuclear hydroxylation and β-oxidation of the side chain.

Further evidence of β-oxidation by a microorganism has been presented by Webley, Duff & Farmer (1955), who studied the metabolism of ω-phenyl-substituted fatty acids by *Nocardia opaca* Waksman & Henrici. Microbiological nuclear hydroxylation of these acids was also reported by these workers, and of 4-chlorophenoxyacetic acid by Evans & Smith (1954).

The importance of β-oxidation in the plant-growth-regulating activity of the phenoxyalkylcarboxylic acids has been clearly demonstrated by Fawcett, Ingram & Wain (1954) and has led to the development of highly selective weed-killers (Wain, 1954). Though the side chains of these acids are β-oxidized by the flax plant (Fawcett *et al.* 1954), Fawcett, Spencer & Wain (1955) found no indication from fungitoxicity tests that this degradation could be effected by the fungi *Botrytis fabae* Sardiña and *Pythium ultimum* Trow.

In addition to their use as plant-growth regulators phenoxyalkylcarboxylic acids have been studied as possible systemic fungicides (Fawcett *et al.* 1955). The present paper describes the metabolism of four members of this series by *A. niger*.

* Part 1: Byrde, Harris & Woodcock (1956).

EXPERIMENTAL

Materials and reference compounds

Phenoxyacetic acid. This was prepared by the condensation of sodium phenoxide and ethyl bromoacetate. It crystallized from benzene–light petroleum (b.p. 60–80°) in prisms, m.p. 97–98°. Fritzsche (1879) found m.p. 96°.

β-Phenoxypropionic acid. β-Propiolactone (3·6 g.) was added dropwise with stirring during 5 min. to a boiling solution of phenol (4·7 g.) and NaOH (2 g.) in water (30 ml.). The solution was boiled for a further 20 min., cooled, acidified and extracted with ether. The acid crystallized from aqueous methanol in prisms, m.p. 94–95°. (Found: C, 64·9; H, 6·0. Calc. for $C_9H_{10}O_3$: C, 65·0; H, 6·0%.) Bischoff (1900) found m.p. 97·5–98°.

γ-Phenoxy-n-butyric acid. Crude γ-phenoxybutyronitrile (11·2 g.), obtained by refluxing a solution of γ-phenoxypropyl chloride (11·4 g.) (see below) and sodium cyanide (4·4 g.) in ethanol (70 ml.) overnight, was hydrolysed by refluxing for 18 hr. with a solution of KOH (5·4 g.) in water (5 ml.) and ethanol (70 ml.). The acid, crystallized from light petroleum (b.p. 40°), had m.p. 64–65°. (Found: C, 66·6; H, 6·7. Calc. for $C_{10}H_{12}O_3$: C, 66·7; H, 6·7%.) Lohmann (1891) found m.p. 60°.

γ-Phenoxypropylmalonic acid. A solution of sodium phenoxide, prepared from phenol (18·8 g.) and sodium (4·6 g.) in ethanol (50 ml.), was added dropwise over a period of 1 hr. to a boiling solution of 1-bromo-3-chloropropane (40 ml.) in ethanol (40 ml.). After heating under reflux for a further 4 hr., the mixture was cooled, diluted with water and extracted with ether. After removal of the solvent, distillation of the residue gave γ-phenoxypropyl chloride, b.p. 90–100°/0·5 mm. (30 g.). This was refluxed for 6 hr. with a solution of ethyl sodiomalonate, prepared from diethyl malonate (33·6 g.) and sodium (4·8 g.) in ethanol (120 ml.). After the addition of 40% NaOH (40 ml.) the mixture was heated for a further 30 min., cooled, and acidified with conc.

HCl. The product was extracted with ether, and the extract washed with water and dried (Na_2SO_4). Removal of the solvent gave a product, m.p. 75–80°, which after drying at 100° crystallized from ether–light petroleum (b.p. 60–80°) in prisms (15 g.), m.p. 110–112°. (Found: C, 60·6; H, 5·7. Calc. for $C_{12}H_{14}O_5$: C, 60·5; H, 5·9%.) Gabriel (1892) found m.p. 75–80°.

δ-Phenoxyvaleric acid. γ-Phenoxypropylmalonic acid (15 g.) was heated at 140° for 1 hr. The product crystallized from light petroleum (b.p. 60–80°) in plates, m.p. 66°. (Found: C, 67·9; H, 7·1. Calc. for $C_{11}H_{14}O_3$: C, 68·0; H, 7·2%.) Gabriel (1892) found m.p. 66°.

o-Hydroxyphenoxyacetic acid. Catechol (11 g.) was added to a solution of sodium (2·3 g.) in ethanol (150 ml.) and refluxed with ethyl bromoacetate (11·2 ml.) for 18 hr. After hydrolysis of the ester by refluxing for a further 1 hr. with 40% NaOH solution (26 ml.) the required acid was isolated, with $NaHCO_3$ solution. Several crystallizations from acetone–benzene (with charcoal treatment) gave a product, m.p. 135–136°. (Found: C, 57·4; H, 4·9. Calc. for $C_8H_8O_4$: C, 57·1; H, 4·8%.) Carter & Lawrence (1900) found m.p. 152°.

p-Hydroxyphenoxyacetic acid. This was prepared from quinol, as described above for the corresponding *ortho* compound. It crystallized from acetone–benzene in prisms, m.p. 151–152°. (Found: C, 57·0; H, 4·7%.) Carter & Lawrence (1900) found m.p. 152°.

β-(p-Hydroxyphenoxy)propionic acid. This was prepared, with quinol and β-propiolactone, by a procedure similar to that described for β-phenoxypropionic acid. It crystallized from acetone–benzene (with charcoal) in minute prisms, m.p. 169·5–170·5. (Found: C, 59·1; H, 5·4. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5%.) Gottesman (1933) found m.p. 168–169°.

γ-(p-Hydroxyphenoxy)-n-butyric acid. This was prepared by hydrolysis of the corresponding nitrile. It crystallized from water (with charcoal) in aggregates of prisms, m.p. 121–122°. (Found: C, 60·8; H, 6·1. Calc. for $C_{10}H_{12}O_4$: C, 61·2; H, 6·1%.) Sobotka & Austin (1952) found m.p. 120°.

METHODS

Replacement culture technique and isolation of metabolites. The following minor modifications were made to the general methods previously described (Byrde *et al.* 1956):

Penicillin culture flasks were used throughout, with 500 ml. of substrate.

As a spray reagent for identifying acidic spots on paper chromatograms, B.D.H. Universal Indicator (Long, Quayle & Stedman, 1951) was used instead of bromocresol green. The former has the advantage that it can be used on papers run in an ammoniacal solvent.

Owing to the presence of more than one product from the metabolism by *A. niger* of the acids studied, separation was always effected by large-scale paper chromatography as described previously (Byrde *et al.* 1956).

Fungitoxicity in vitro. Fungistatic activity was assessed by rate of mycelial growth of *A. niger* on an agar medium in which the compounds under test were incorporated. The following medium was used: glucose, 50 g.; KNO_3, 5 g.; KH_2PO_4, 3·4 g. (equivalent to 0·025 M); $MgSO_4,7H_2O$, 1 g.; Na_2SO_4, 1 g.; agar, 20 g.; water to 1 l. It was found unnecessary to add trace metals for adequate growth.

The medium was dispensed in 50 ml. lots in 100 ml. flasks, autoclaved at 15 lb./in.² for 15 min., and held at 45–50° in a water bath. Phosphoric acid was then added to give a final phosphate concentration of 0·05 M. The pH of this medium was approximately 2·2, sufficiently low to ensure that the acids were predominantly in the undissociated form (pK_a of phenoxyacetic acid, 3·12). The acids were incorporated as ethanolic solutions of appropriate concentration by means of a sterile pipette; the final concentration of ethanol in the medium was adjusted to 1% (v/v) throughout.

Four replicate Petri dishes were poured from each of the flasks, which included a control. On cooling, the plates were inoculated at a central point with a 4·5 mm. disk cut from a day-old culture of *A. niger*, and incubated at 25°. Mycelial growth along two predetermined diameters at right angles was measured on the second and fourth days after inoculation. The mean growth rate (mm./day) over this arbitrary period was used to measure fungistatic activity, and was subjected to statistical analysis. Where acids were tested at a series of concentrations, toxicity data were computed by the probit method as adapted for quantitative responses (Finney, 1952, p. 183).

RESULTS

Identification of metabolites

The metabolism of ω-phenoxy-*n*-alkylcarboxylic acids by *A. niger* gave hydroxy acids detected on paper chromatograms with 1% (w/v) *p*-nitrobenzene diazonium fluoroborate solution in 20%

Table 1. *Metabolites of ω-phenoxy-*n*-alkylcarboxylic acids incubated with* A. niger

Conditions were as described in the text and by Byrde *et al.* (1956). β-(*o*-Hydroxyphenoxy)propionic acid was identified by its R_F value, the remainder by mixed m.p.'s.

Substrate	Principal metabolites: Identity	M.p.	R_F
Phenoxyacetic acid	*o*-Hydroxyphenoxyacetic acid	130–135°	0·45
	p-Hydroxyphenoxyacetic acid	140–150	0·23
β-Phenoxypropionic acid	β-(*o*-Hydroxyphenoxy)propionic acid	—	0·59
	β-(*p*-Hydroxyphenoxy)propionic acid	168–169	0·43
γ-Phenoxy-*n*-butyric acid	*o*-Hydroxyphenoxyacetic acid	125–130	0·42
	p-Hydroxyphenoxyacetic acid	145–150	0·24
δ-Phenoxy-*n*-valeric acid	β-(*o*-Hydroxyphenoxy)propionic acid	—	0·58
	β-(*p*-Hydroxyphenoxy)propionic acid	169–171	0·43

(w/v) aqueous sodium acetate. This reagent gave a dark-brown colour with *o*-hydroxyphenoxy-acetic and -propionic acids and a light reddish-brown colour with the corresponding *p*-hydroxy acids. Table 1 summarizes the identification of the principal metabolites. In a typical experiment in which 76 mg. of phenoxyacetic acid in 500 ml. of 0·01 M-Na_2HPO_4 was incubated with *A. niger*, 31 mg. of *p*-hydroxyphenoxyacetic acid and 7 mg. of *o*-hydroxyphenoxyacetic acid were isolated.

Hydroxylation with a model system

The ascorbic acid system of Udenfriend, Clark, Axelrod & Brodie (1954) used previously (Byrde *et al.* 1956) was again employed to investigate the hydroxylation of phenoxyacetic acid. A solution of this acid (0·09 g.), ascorbic acid (0·25 g.), $FeSO_4$, $7H_2O$ (0·04 g.) and sodium ethylenediaminetetra-acetate (0·23 g.) in 0·2 M phosphate buffer, pH 6·8 (30 ml.), was aerated at 37° for 2 hr. The solution was then acidified with HCl, extracted with ether and acidic products were re-isolated by shaking with $NaHCO_3$ solution. Chromatographic examination of the acidified bicarbonate-soluble fraction gave spots identical with phenoxyacetic acid (R_F 0·51), *o*- and *p*-hydroxyphenoxyacetic acids (R_F values 0·46 and 0·28 respectively) and a further unidentified spot (R_F 0·65) which gave a dark-brown colour with the diazonium reagent. The *o*-hydroxy compound, which had been present in the fungal extracts to only a small extent compared to the *para*-isomer, here appeared to be the major product.

Fungitoxicity in vitro

Table 2 shows the mean growth rate (mm./day) of cultures of *A. niger* in the presence of unsubstituted and hydroxylated phenoxyacetic and β-phenoxypropionic acids. The *p*-hydroxy derivative of each acid, even at the relatively high concentrations tested, produced no significant inhibition of fungal growth. Probit analysis of the data for the unsubstituted and *o*-hydroxy acids was carried out, and the results are summarized in Table 3. The ratio of the potency to *A. niger* of phenoxyacetic acid to that of its *o*-hydroxy derivative was 7·6:1; no overall figure for potency can be quoted for the corresponding β-propionic acids, owing to non-parallelism of dose–response lines, but the ED_{50} values were in the ratio of 17:1, the unsubstituted acid again being the more toxic.

Inspection of the data of Table 2 shows that, with the agar-plate method described above, it was possible to detect differences in growth which were of the order of 10 % of that of the control plates to which ethanol only had been added. By contrast, in the studies on ω-(2-naphthyloxy)-*n*-alkylcarboxylic acids where mycelial growth in liquid medium was estimated gravimetrically, the differences between

Table 2. *Mean growth rate of* A. niger *mycelium in the presence of phenoxyacetic and β-phenoxypropionic acids and their* o- *and* p-*hydroxy derivatives*

Conditions were as described in the text.

Test no.	Compound added	10^4 × concn. (M)	Mean growth rate (mm./day)
1	Phenoxyacetic acid	20·0	4·92
		10·0	8·31
		5·0	9·44
		2·5	9·94
	o-Hydroxyphenoxyacetic acid	100·0	7·00
		50·0	9·25
		25·0	9·56
	p-Hydroxyphenoxyacetic acid	100·0	10·88
		50·0	10·83
	None	—	10·38
	Least significant difference between means	(P=0·05)	0·98
2	β-Phenoxypropionic acid	10·0	0·25
		5·0	2·38
		2·5	9·19
		1·25	11·06
	β-(*o*-Hydroxyphenoxy)propionic acid	50·0	6·50
		25·0	9·13
		12·5	10·44
	β-(*p*-Hydroxyphenoxy)propionic acid	50·0	9·50
		25·0	11·44
	None	—	10·69
	Least significant difference between means	(P=0·05)	1·42

Table 3. *Dose–response data derived from Table 2*

Test no.	Compound	Equation of probit regression line	10^4 ED_{50}* (molar)	Slope of probit regression line	Relative potency*
1	Phenoxyacetic acid	$Y = 8{\cdot}48 - 2{\cdot}65x$	20·6 (17·6–24·1)	$-2{\cdot}65 \pm 0{\cdot}39$	100·0
	o-Hydroxyphenoxyacetic acid	$Y = 10{\cdot}81 - 2{\cdot}65x$	156·3 (127·6–191·5)	$-2{\cdot}65 \pm 0{\cdot}39$	13·2 (10·8–16·0)
2	β-Phenoxypropionic acid	$Y = 8{\cdot}39 - 5{\cdot}91x$	3·8 (3·2–4·2)	$-5{\cdot}91 \pm 0{\cdot}91$	†
	β-(*o*-Hydroxyphenoxy)propionic acid	$Y = 9{\cdot}58 - 2{\cdot}54x$	63·7 (49·8–148·5)	$-2{\cdot}54 \pm 0{\cdot}88$	†

* Approximate 5% fiducial limits are quoted in parentheses.
† No estimate possible, owing to non-parallelism of lines. Ratio of ED_{50} values, 17:1.

transformed treatment means required to be about 20% of the control in order to attain statistical significance (Byrde *et al.* 1956; Tables 2 and 3). The solid–medium technique was thus more sensitive, and also obviated the need for an angular transformation of the data.

DISCUSSION

The results obtained with the phenoxy-*n*-alkylcarboxylic acids followed the general pattern of those in the 2-naphthyloxy series, both nuclear hydroxylation and β-oxidation of the side chain being demonstrated. Whereas hydroxylation by *A. niger* was limited to the 6-position in the naphthalene nucleus, it occurred in both the *para* and to a lesser extent in the *ortho* position in the benzene nucleus. Parke & Williams (1956), studying the detoxication of aniline by animals, also found that such preferential *para*hydroxylation occurred in the majority of species. Hydroxylation of phenoxyacetic acid with the Fe^{2+} ions–ascorbic acid system of Udenfriend *et al.* (1954), on the other hand, took place primarily in the *ortho* position, though *para*-substitution also occurred to some extent.

Whereas γ-phenoxy-*n*-butyric and δ-phenoxy-*n*-valeric acids were metabolized, forming mixtures of *o*- and *p*-hydroxyphenoxyacetic and *o*- and *p*-hydroxy-β-phenoxypropionic acids respectively, the side chain of the two latter acids remained intact. This resistance of the side chain to further oxidation has already been noted with the corresponding β-(6-hydroxy-2-naphthyloxy)propionic acid, but this is in sharp contrast with the results of Fawcett *et al.* (1954), who found that β-phenoxypropionic acid is converted into phenol in the flax plant.

The biological-assay data presented show that the hydroxylation of phenoxyacetic and β-phenoxypropionic acids is accompanied by a decrease in toxicity to the fungus. It was also found that the *o*-hydroxy acids were both more toxic than the corresponding *p*-hydroxy compounds, probably due to the more lipophilic nature of the former.

The significant difference between the slopes of the dose–response lines for β-phenoxypropionic and β-(*o*-hydroxyphenoxy)propionic acids suggests a dissimilar mode of action (Finney, 1952, p. 124). A difference in slope of dose–response lines after hydroxylation was also noted in the naphthyloxy acid series (Byrde *et al.* 1956). Recent findings, however, suggest that caution is needed in interpreting such differences in slope as necessarily implying dissimilarity in mode of action of fungicides (McCallan, unpublished work).

SUMMARY

1. By the use of a replacement culture technique it was demonstrated that phenoxyacetic and β-phenoxypropionic acids were hydroxylated in the *para* and to a lesser extent the *ortho* positions by *Aspergillus niger*, both products in each case being less toxic to the fungus than the original acid.

2. γ-Phenoxy-*n*-butyric acid likewise gave mainly *p*-hydroxyphenoxyacetic acid by hydroxylation and β-oxidation.

3. δ-Phenoxy-*n*-valeric acid was similarly metabolized to β-(*p*-hydroxyphenoxy)propionic acid (and probably also to the *o*-hydroxy acid).

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The Oxidative Pathway of Carbohydrate Metabolism in *Escherichia coli*

5. ISOLATION AND IDENTIFICATION OF RIBULOSE PHOSPHATE PRODUCED FROM 6-PHOSPHOGLUCONATE BY THE DEHYDROGENASE OF *E. COLI**

By DWIGHT B. McNAIR SCOTT and S. S. COHEN
The Children's Hospital of Philadelphia (Department of Pediatrics) and the Department of Biochemistry of the University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania

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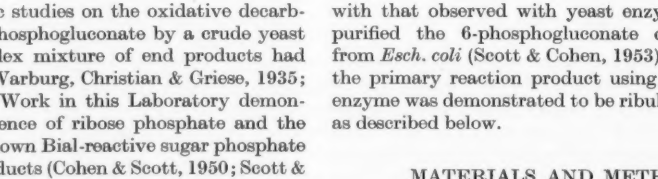
In early enzymic studies on the oxidative decarboxylation of 6-phosphogluconate by a crude yeast system, a complex mixture of end products had been obtained (Warburg, Christian & Griese, 1935; Dickens, 1938). Work in this Laboratory demonstrated the presence of ribose phosphate and the presence of unknown Bial-reactive sugar phosphate among these products (Cohen & Scott, 1950; Scott & Cohen, 1951). The use of a considerably purified enzyme system from yeast permitted the further dissection of this reaction sequence by Horecker, Smyrniotis & Seegmiller (1951). These workers thus discovered the formation of ribulose 5-phosphate as a primary product of the oxidative decarboxylation of 6-phosphogluconate, and the subsequent isomerization of ribulose 5-phosphate to ribose 5-phosphate.

Subsequent work on this system in our Laboratory was concerned primarily with the role of this pathway in ribose formation in *Escherichia coli* under conditions of normal growth and virus infection. Nevertheless, it appeared desirable to demonstrate unequivocally that the oxidation of 6-phosphogluconate by bacterial enzymes was comparable with that observed with yeast enzymes. Having purified the 6-phosphogluconate dehydrogenase from *Esch. coli* (Scott & Cohen, 1953), the nature of the primary reaction product using this bacterial enzyme was demonstrated to be ribulose phosphate as described below.

* Part 4: Scott (1956).

MATERIALS AND METHODS

The barium salt of 6-phosphogluconic acid was prepared from synthetic glucose 6-phosphate by the method of Seegmiller & Horecker (1951). Phosphogluconate (PG) was determined by the reduction of triphosphopyridine nucleotide (TPN) in the presence of phosphogluconic dehydrogenase. The barium salt of ribose 5-phosphate was prepared by the method of Groth, Mueller & LePage (1952) and always contained about 11% of ribulose 5-phosphate. Sodium pyruvate was prepared immediately before use by neutralization of freshly distilled pyruvic acid. Glycylglycine buffer and tris buffer (aminotrishydroxymethylmethane) were obtained from Sigma Chemical Co. TPN was prepared by the method of LePage & Mueller (1949) and was measured by reduction in the presence of PG and PG dehydrogenase. It was 74% pure. Pure ribulose was regenerated from the *o*-nitrophenylhydrazone of ribulose by treatment with freshly distilled benzaldehyde in the presence of benzoic acid. This derivative had been prepared from ribulose obtained



by chemical or enzymic isomerization of D-arabinose (Glatthaar & Reichstein, 1935; Cohen, 1953). After filtration, the solution containing ribulose was extracted with chloroform and ether and taken to a syrup by vacuum distillation. D-Arabinose was obtained from Pfanstiehl Chemical Co.

Lactic dehydrogenase was prepared from rabbit muscle according to the method of Cori, Slein & Cori (1948) and Kornberg & Pricer (1951). After several precipitations with $(NH_4)_2SO_4$ the activity in oxidizing reduced diphosphopyridine nucleotide was 41 μmoles/min./mg. of protein and in oxidizing reduced triphosphopyridine nucleotide (TPNH) was about 0·6 μmole/min./mg. This enzyme preparation did not contain phosphatase, pentose phosphate isomerase, ribose phosphate-splitting enzyme or PG dehydrogenase.

Acid phosphatase was prepared from frozen semen by two precipitations with saturated $(NH_4)_2SO_4$ so that it gave neither a fructose reaction with carbazole nor a pentose reaction with Bial reagent. This enzyme preparation contained no phosphorus and had no pentose isomerase activity. Incubation of this enzyme with glucose 6-phosphate, PG, or ribose 5-phosphate in acetate buffer at pH 5·0 removed 100% of the phosphate in 60 min. at 37°; Mg^{2+} ions were inhibitory.

Pentose isomerase was prepared from cultures of *Esch. coli* Ba 15 (Cohen, 1953) grown in medium containing D-arabinose. The wet pellet of bacteria was ground with alumina and extracted with 0·05 M tris buffer at pH 8·0. Nucleic acids were precipitated with $MnCl_2$. The precipitate formed in 63% saturated $(NH_4)_2SO_4$ was discarded. The protein precipitated at pH 4·7 contained an active pentose isomerase and also phosphopentose isomerase. An activating effect of Mn^{2+} ions could be detected on this precipitated pentose isomerase; tris and ethylenediaminetetraacetic acid were inhibitory. The enzyme was used in glycylglycine buffer at pH 7·2. When either pure ribulose or pure D-arabinose was incubated with the enzyme in this buffer an equilibrium mixture resulted, containing approximately 20% of ribulose and 80% of arabinose.

Phosphogluconate dehydrogenase was prepared from 500 ml. of *Esch. coli* B, grown to a turbidity of 1400 as measured on a Klett photoelectric colorimeter fitted with a 420 mμ. filter. The viable count was 7×10^9 bacteria/ml. The aerated liquid synthetic medium contained increased amounts of $(NH_4)_2SO_4$ and glucose as described earlier (Scott & Cohen, 1953). The centrifuged cells were washed with cold saline. The moist pellet weighed 4·3 g. and was ground for several minutes in a chilled mortar with 2·5 times the weight of Al_2O_3. The resulting paste was extracted successively with 50 ml. and 25 ml. portions of cold water. The addition of 5 ml. of 0·5 M-$MnCl_2$ to the combined extracts precipitated most of the nucleic acids after 17 hr. at 4°. The precipitate was discarded. Saturated $(NH_4)_2SO_4$ (pH 8·0) was added to the supernatant fluid to a saturation of 62% (by vol.). This precipitate contained most of the glucose 6-phosphate dehydrogenase activity. The supernatant fluid was brought to pH 5·0 by addition of 0·5 M acetate buffer at pH 3·8. This precipitate was either discarded or used for the preparation of pentose phosphate isomerase. Addition of more acetate buffer to the supernatant fluid to bring the pH to 4·3 produced a precipitate which contained most of the PG dehydrogenase and no glucose 6-phosphate dehydrogenase. This precipitate was dissolved in 6 ml. of M/8 glycylglycine buffer, pH 7·4. Dialysis of this solution overnight against cold-running distilled water caused the formation of a further precipitate. The precipitate, which was dissolved in glycylglycine buffer, pH 7·4, had all the PG dehydrogenase activity and slight isomerase, transketolase, and phosphatase action.

The supernatant solution after dialysis of the PG dehydrogenase contained pentose phosphate isomerase with activity of 5 μmoles/30 min./mg. of protein. This preparation had no PG dehydrogenase activity, low pentose phosphate degrading activity and slow phosphatase action. This enzyme was used with ribose 5-phosphate to prepare mixtures of ribose 5-phosphate and ribulose 5-phosphate in order to determine the separation of these two components by elutions from columns.

Separation of the phosphate compounds was carried out by adsorption on a column (20 mm. × 135 mm.) of Dowex 1, washed with HCl and then with water until no further Cl^- ions were eluted. Elution was effected by increasing concentrations of monochloroacetic acid from 0·001 M to M (Groth *et al.* 1952).

Pentose and pentose phosphate were determined by a modification (Miller, Golder & Miller, 1951) of the Bial reaction. Ribulose and ribulose phosphate were determined by the carbazole–cysteine–H_2SO_4 method of Dische & Borenfreund (1951) and also by the decrease in Bial-reacting substance after treatment with KOH. Ribose and ribose phosphate were determined from the Bial reaction after treatment of the solution with KOH. Spectra of the Bial reactions between 400 and 700 mμ., especially the 540 mμ. peak indicative of free ribulose, were very useful in characterizing the products (Cohen, 1953).

Phosphates were determined by the method of King (1932). Digestion of samples for determination of total phosphorus was performed with perchloric acid.

RESULTS

The reaction mixture for the degradation of 6-phosphogluconate was a solution (52 ml.) containing purified PG dehydrogenase, 0·0065 M PG (340 μ-moles), 0·0102 M sodium pyruvate, 0·015 M-$MgCl_2$ and 0·05 M glycylglycine buffer, pH 7·6. A portion (2 ml.) was removed for analysis and the reaction was begun in the remaining 50 ml. of solution by adding 0·5 ml. of lactic dehydrogenase and 4 μmoles of TPN in 0·4 ml. This mixture was allowed to react at 37° for 60 min. Seven samples of 0·2 ml. each were removed at intervals for analysis. After 1 hr. approx. 320 μg. of ribulose/ml. had been produced, i.e. 15 mg., or 100 μmoles, indicating that 30% of the phosphogluconate had reacted.

The mixture was chilled and put directly on a column of Dowex 1 (acid form) and washed through with 100 ml. of water. Elution was effected with monochloroacetic acid, starting at 0·001 M and increasing in concentration. The first washing contained protein, 140 μg. of free ribulose and no P. At 0·04 M acid, Bial-reacting material and organic P started to be eluted and came off slowly in fractions 118–152. Bial-reactive and carbazole-unreactive material appeared in fractions 118–121,

amounting to approximately 0·5 mg. of pentose. Fractions 121–151 contained 15 mg. of ribulose, and 3·36 mg. of P of which 0·26 mg. was inorganic P. Thus the ratio ribulose/organic P was 1. Fractions 121–131 contained approx. 1 mg. of pentose phosphate; thus there was a slight pentose phosphate isomerase activity during the reaction, such that approx. 7 % of the reaction product was pentose phosphate other than ribulose phosphate.

In fractions 152–155 pyruvate was eluted, and from 164 to 178, 0·13 mg. of organic P of unknown composition. Fractions 179–182 gave 7·55 mg. of organic P and contained the unchanged PG, which was measured by the action of PG dehydrogenase. Fractions 183–185 contained 0·37 mg. of Bial-reactive material and 0·22 mg. of organic P, and was probably TPN.

The combined fractions 121–151, consisting of 320 ml. minus 8 ml. taken for various analyses, was extracted three times with ether to remove the monochloroacetic acid and was then concentrated *in vacuo* to 17 ml. The acid solution was neutralized to pH 5·0 with KOH. To this solution was added 0·4 ml. of acid phosphatase and the mixture was incubated at 37° for 45 min., when the total P (3·36 mg. or 108 μmoles) was inorganic. Analysis indicated 10·5 mg. of ribulose and 1·6 mg. of ribose or 80 μmoles of pentose.

The mixture was brought to pH 7·0 with NaOH, and 2·3 ml. of pentose isomerase enzyme was added. The mixture was incubated at 37° for 2 hr., at which time the carbazole reaction was one-half of that of the mixture before addition of the isomerase. The Bial reaction at first increased, and after 1 hr. was equivalent to 12·8 mg. of pentose. After 2 hr. it had decreased, giving a value of 11 mg., so the reaction was stopped by chilling to avoid further destruction of the pentose.

The mixture was then de-ionized by alternate treatment with Dowex 50 in H^+ form and Dowex 1 in CO_3^{2-} form until the phosphate had been removed and the solution had a pH of 7·0. There was a loss of pentose during this desalting procedure, and the 35 ml. of solution now contained 8·8 mg. of pentose. By vacuum distillation the volume was decreased to 3 ml. A slight precipitate was removed by centrifuging and the solution was further concentrated by a stream of dry air to 0·2 ml.

To this was added 0·4 ml. of ethanol containing 6 mg. of sodium acetate and 12·5 mg. of diphenylhydrazine hydrochloride. After standing overnight at 4° a good crop of crystals was obtained. After one recrystallization from ethanol the m.p. was 202–203° (decomp.), and after a second recrystallization, 205–206·5°. The diphenylhydrazone derivative of authentic D-arabinose melted at 204–205°, mixed m.p. 206–206·5° (decomp.). These values were obtained on a Fischer–Johns apparatus and are uncorrected (Cohen, 1953).

DISCUSSION

The reactions used in the degradation of phosphogluconate and the identification of the final product are given below:

(*a*) TPN + 6-phosphogluconate → TPNH + 90 % ribulose phosphate + 9 % ribose phosphate + 1·6 % unknown pentose phosphate.

(*b*) TPNH + pyruvate $\xrightarrow[\text{dehydrogenase}]{\text{lactic}}$ TPN + lactic acid.

(*c*) Pentose phosphates $\xrightarrow[\text{phosphatase}]{\text{acid}}$ pentoses + inorganic phosphate.

(*d*) Ribulose $\xrightarrow[\text{isomerase}]{\text{pentose}}$ D-arabinose.

(*e*) D-Arabinose + diphenylhydrazine HCl → D-arabinose diphenylhydrazone.

It has been demonstrated recently that ribulose 5-phosphate may be converted into xylulose 5-phosphate by a pentulose phosphate epimerase (Hochster, 1955; Stumpf & Horecker, 1956). It may be asked therefore whether xylulose 5-phosphate might have been the primary product of enzymic degradation of phosphogluconate and had been converted into ribulose 5-phosphate in this system. The following considerations argue against this possibility.

(1) The configuration of the hydroxyl groups of ribulose 5-phosphate at C-3 and C-4 are *cis*, as they are in the parent C-4 and C-5 of 6-phosphogluconate, whereas these hydroxyl groups are *trans* in xylulose 5-phosphate.

(2) The equilibrium mixture has been shown, with an enzyme from *Lactobacillus pentosus* (Stumpf & Horecker, 1956), to be a 45:55 mixture of xylulose 5-phosphate and ribulose 5-phosphate. If the epimerase had been present in the dehydrogenase preparation, an approximately equimolecular mixture of these compounds should have been obtained. Two peaks of pentulose phosphate were not observed in elution from the column, though the pentulose phosphate generated in the reaction mixture was quantitatively recovered from the column.

(3) On dephosphorylation a 70 % recovery of ribulose was observed. That this pentulose was indeed ribulose may be concluded from the following evidence: (*a*) The spectrum of ribulose in the Bial reaction is different from that of xylulose (Stumpf & Horecker, 1956). Our material gave the spectrum of ribulose in this reaction. (*b*) The development of colour in the cysteine–carbazole reaction is much faster for ribulose than for xylulose (Cohen, 1953; Stumpf & Horecker, 1956). The pentulose reacted in this test as ribulose. (*c*) The pentose isomerase used

is specific for compounds of the ribulose configuration and does not act on xylulose (Cohen, 1953). All the pentulose detectable in the cysteine–carbazole reaction was converted into aldopentose. It may be noted that the specific enzyme is an elegant tool in distinguishing the pentuloses and in permitting the estimation of one in the presence of the other. The same enzyme may also be applied to the specific estimation of L-fucose, which is converted into L-fuculose (Green & Cohen, 1956).

Since no evidence was obtained for pentulose other than ribulose, and this compound was isolated in the form of pentulose phosphate as the only product of the degradation of 6-phosphogluconate, it has been concluded that the primary product which could be isolated after degradation of PG by the dehydrogenase of *Esch. coli* was ribulose phosphate, as has been observed for the PG dehydrogenases of yeast and liver.

SUMMARY

1. A purified 6-phosphogluconate dehydrogenase was prepared from extracts of *Escherichia coli* B. This preparation was very low in phosphatase, pentose phosphate isomerase and transketolase or other ribose phosphate-splitting enzymes.

2. After action of the enzyme on phosphogluconate, the reaction mixture was fractionated on an ion-exchange column. Ribulose 5-phosphate was isolated in high yield. It was dephosphorylated, and the free ribulose was enzymically converted into D-arabinose, which was isolated as the characteristic D-arabinose diphenylhydrazone.

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Studies on the Composition of Lipids of the Rabbit

By J. H. FUTTER and F. B. SHORLAND
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

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Of the pasture-fed animals the wild rabbit has a special interest, because linolenic acid, the main dietary fatty constituent, forms over 40% of the fatty tissues as compared with less than 1% found in the depot fats of ruminants (Shorland, 1953). Earlier studies on pasture-fed horses, which also contain a high proportion (about 16%) of linolenic acid in their fatty tissues (Brooker & Shorland, 1950; Gupta & Hilditch, 1951) have shown that this acid is differentially distributed between the tissue lipids (Shorland, Bruce & Jessop, 1952). Whereas the glyceride fractions contain trienoic (linolenic) acid as the main C_{18} unsaturated fatty acid, in the phospholipid C_{18} unsaturated fatty acids dienoic constituents predominate. In this paper we report a similar study on the lipids of the rabbit.

EXPERIMENTAL

The rabbits used in this work were trapped on 6 and 7 June 1951 (mid-winter), in the Guavas Forest area, near Waipawa, Hawkes Bay, New Zealand, by officers of the Animal Ecology Section of the Department of Scientific and Industrial Research, New Zealand. There were twenty-five males, average wt. 1575 S.D. ± 135 g., and eighteen females, average wt. 1499 S.D. ± 160 g. A few of the females were in the early stages of pregnancy and almost all of the rabbits

had a thin layer of fatty tissue in the abdominal cavity. In rabbits trapped six months earlier such fat was not visible, and at that stage many of the females had young litters.

The traps were set in the evening and the rabbits collected at dawn. With the least possible delay the following tissues were removed: liver, kidney, longissimus dorsi muscle and fatty tissue from the abdominal cavity. The corresponding material from the males and females was bulked separately, finely sliced, and sterilized by dropping into boiling ethanol and holding at boiling point for about 15 min. With the longissimus dorsi muscle the whole sample was not used, but a representative portion was taken. In addition to the samples just described, yellow abdominal fat was separated from two male rabbits and one female from another collection, to compare this fat with the white fat usually encountered.

The extraction of the lipids from the tissues, and the separation of glyceride and phospholipid fractions, were carried out essentially as described by Shorland *et al.* (1952). Small amounts of phospholipids remaining in the glycerides were, however, removed by passing a chloroform solution of the fraction through a silica gel column as described by Borgström (1952).

By elution with chloroform it was possible to recover almost completely the non-phospholipid constituents while retaining the phospholipids on the column. The phospholipids were subsequently removed with methanol and added to the phospholipids earlier separated by their insolubility in acetone. The characteristics of the lipids before separation into phospholipids and glycerides are shown in Table 1.

In previous work it has been customary to refer to the acetone-soluble fraction as 'glycerides' (cf. Hilditch & Shorland, 1937), though it is well appreciated that such fractions contain also cholesterol esters and perhaps wax esters. However, it appears that the unsaponifiable matter in animal-tissue fats consists largely of cholesterol present mainly in the free form (cf. Hilditch & Shorland, 1937; Bloor, 1943). It is reasonable to suppose therefore that in the present investigation the acetone-soluble fraction will consist mainly of glycerides. It is proposed for convenience here to continue to use the term 'glycerides' to designate the acetone-soluble constituents, recognizing that in the present work, and indeed, in work on natural fats generally, the term glycerides does not imply the complete absence of sterol esters, wax esters and unsaponifiable constituents.

The ester-fractionation analyses were carried out as previously described by Shorland *et al.* (1952). In determining the mean unsaturation of the C_{20} unsaturated acids by the customary procedure of graphical extrapolation (cf. Hilditch, 1956) it became apparent that some polymerization had occurred during distillation. The mean unsaturation values shown in Table 2 are therefore probably lower than in the original lipid. The polymerization of the methyl esters of the C_{22} unsaturated acids was even more extensive. To calculate the proportions of these acids it was therefore necessary to assign arbitrarily the same mean unsaturation as was found for the C_{20} unsaturated acids. Furthermore, because of the small amounts of lipids (5–10 g.) available in the non-fatty tissues, the traces of C_{22} unsaturated acids were perhaps not detected in all instances, as it was not practicable to carry the ester fractionation far enough. The problem now described is, however, generally inherent in the ester-fractionation technique, and hence it is not uncommon to assign the C_{20} and above unsaturated acids to the C_{20}–C_{22} group even though there is no direct evidence from the saponification equivalents of the methyl ester fractions for acids of higher molecular weight than C_{20}. Examples of this kind are to be found in recent publications (cf. Hilditch, 1956). Therefore, in Table 2 the non-detection of C_{22} unsaturated acids does not necessarily imply their absence, but indicates merely that such acids, if present, occur in only minor proportions.

In calculating the proportions of saturated acids of higher molecular weight than stearic acid, it has been assumed that arachidic acid only is present, though the probable occurrence of acids of still higher molecular weight is fully recognized.

The composition of the C_{18} unsaturated acids was determined by the Brice & Swain (1945) procedure, purified concentrates of methyl esters obtained during ester fractionation being used (see Table 2). The composition of the C_{20} unsaturated acids was not studied in detail owing to the difficulty in obtaining purified fractions. Nevertheless, through the application of the spectrophotometric procedure as described by Shorland & Johannesson (1951), it was possible to estimate that the C_{20} unsaturated acids of the female liver 'glycerides' contained 14·7% of pentaene and 22% of tetraene constituents, whereas for the liver phospholipid the corresponding values were 14·9 and 47·4% respectively. The absorption at 372 mμ. after alkali isomerization indicated the presence of some 1–2% of hexaene acids in the C_{20} fractions, but the proportions of

Table 1. *Characteristics of lipid fractions*

The percentage of phospholipid was calculated from the P content by multiplying by 25. The free fatty acids are expressed as oleic acid, and the neutral fat was determined as the difference between the total lipids and the sum of the free fatty acids and phospholipids. M., Male; F., female.

Origin of fat	Average wt. (g.)	Lipids in tissue (%)	Phospholipids in lipids (%)	Unsaponifiable matter in lipids (%)	Free fatty acids in lipids (%)	Neutral fat in lipids (%)
Abdominal cavity M.	6·2	86·0	Trace	0·2	Trace	100·0
F.	17·6	79·6	Trace	0·5	Trace	100·0
Liver M.	40·6	4·9	40·5	13·9	9·3	50·2
F.	29·1	4·7	39·0	18·5	2·8	58·2
Kidney M.	11·8	3·1	31·0	23·1	4·3	64·7
F.	10·3	5·0	29·2	12·7	3·2	67·6
Longissimus dorsi M.	—	1·9	22·3	20·3	8·4	69·3
F.	—	1·5	35·7	16·2	1·3	63·0

Table 2. *Fatty acid composition of glycerides and phospholipids from the fatty tissues, liver, kidney and muscle of the rabbit*

Fatty acids are expressed as a percentage (w/w). The figures in parentheses indicate the mean unsaturation expressed in terms of hydrogen; the non-detection of C_{22} unsaturated acids is shown by an asterisk. M., Male; F., female.

Origin of fat	Saturated				Unsaturated				
	C_{14}	C_{16}	C_{18}	C_{20}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
				(*a*) Glycerides					
Abdominal cavity M.	1·6	22·2	6·4	0·8	0·4 (2·0 H)	4·4 (2·0 H)	62·9 (4·8 H)	1·3 (4·8 H)	*
F.	1·6	22·2	5·3	0·7	0·4 (2·0 H)	4·1 (2·0 H)	65·7 (5·1 H)	Trace	*
'Yellow fat' Mixed M. & F.	0·2	22·7	6·1	Trace	Trace	7·4 (2·0 H)	63·6 (4·9 H)	Trace	*
Liver M.	—	19·8	18·0	0·6	—	0·6 (2·0 H)	35·6 (4·3 H)	21·8 (5·8 H)	3·6 (5·8 H)
F.	—	21·8	8·9	2·9	Trace	3·2 (2·0 H)	48·0 (3·5 H)	13·3 (6·0 H)	1·9 (as 6·0 H)
Kidney M.	—	22·4	12·0	—	—	1·3 (2·0 H)	49·5 (4·4 H)	14·9 (5·2 H)	*
F.	1·7	21·2	2·4	4·4	—	4·5 (2·0 H)	61·3 (4·4 H)	4·5 (4·4 H)	*
Longissimus dorsi muscle M.	—	14·0	8·7	1·3	—	8·0 (2·0 H)	49·6 (3·4 H)	18·4 (6·3 H)	*
F.	—	18·2	8·0	1·1	—	4·2 (2·0 H)	55·0 (4·3 H)	13·5 (3·7 H)	*
				(*b*) Phospholipids					
Liver M.	—	10·3	13·1	18·5	—	1·3 (2·0 H)	36·0 (3·9 H)	18·2 (5·2 H)	2·6 (as 5·2 H)
F.	—	11·1	26·8	9·1	—	1·1 (2·0 H)	42·1 (3·9 H)	9·8 (6·0 H)	*
Kidney M.	—	20·3	6·0	3·5	—	1·1 (2·0 H)	39·4 (3·6 H)	20·4 (5·5 H)	9·3 (as 5·5 H)
F.	—	18·8	6·6	7·3	—	Trace	43·0 (3·9 H)	18·3 (4·4 H)	6·0 (as 4·4 H)
Longissimus dorsi muscle M.	—	21·2	5·5	9·8	—	5·2 (2·0 H)	34·5 (3·5 H)	16·1 (3·8 H)	7·7 (as 3·8 H)
F.	—	19·1	7·8	8·3	—	3·5 (2·0 H)	35·1 (3·5 H)	20·0 (5·6 H)	6·2 (as 5·6 H)

* Not detected.

hexaene acids in the residual fractions containing C_{22} unsaturated acids could not be estimated satisfactorily owing to the extensive polymerization.

DISCUSSION

The composition of the fatty tissues of the wild rabbit has not hitherto been determined in detail, though Vickery (1928, quoted by Hilditch, 1956), found 5 % of myristic, 23 % of palmitic and 4 % of stearic acids and 68 % of unsaturated acids which were not completely identified. However, from the weight of ether-insoluble polybromides Hilditch (1956) estimated that nearly 20 % of linolenic acid was present. Having regard to the analytical techniques then available, the results indicate a similar fatty acid composition to that shown for the fatty tissues in the present work (see Table 2). The high content (37·7–42·2 %) of linolenic acid in the fatty tissue lipids (see Table 3) shows the ability of the rabbit to store this acid to a level approaching that found (about 60–70 %) in the dietary fat (Shorland, 1953). Comparison made between the fatty tissues of the male and female rabbits and of the yellow fat (see Tables 2 and 3) shows little difference in fatty acid composition, the palmitic acid content (about 22 %) in these samples being somewhat lower than usually found (25–30 %) in mammalian depot fats (cf. Hilditch, 1956).

The presence of substantial amounts (4·5–29·7 %) of C_{20}–C_{22} unsaturated acids in non-fatty tissue lipids, which form the greater part of the total lipids

in the rabbit, raises the question of their origin. It is known that the chief fatty constituent of the diet, linolenic acid, is transformed in the rat into pentaenoic and particularly hexaenoic acids (cf. Widmer & Holman, 1950; Witten & Holman, 1952). In poultry, on the other hand, Reiser (1950, 1951) considers that linolenic acid forms poly-unsaturated acids with from two to six double bonds. These findings are generally supported by Klenk (1955) with labelled acetate in conjunction with linoleic and linolenic acids. He considers that acetate is added at the carboxyl end with the introduction of new double bonds in the divinylmethane pattern.

The evidence as to the nature of the C_{20}–C_{22} unsaturated acids in the non-fatty tissues of the rabbit is somewhat incomplete, though the occurrence of pentaenoic and hexaenoic acids consistent with the conversion of some of the dietary linolenic acid into these constituents was indicated. However, the mechanism for the formation of the less unsaturated C_{20}–C_{22} acids cannot at present be indicated unless, in accordance with Reiser's (1950, 1951) findings on poultry, linolenic acid actually forms the complete range of higher unsaturated acids with from two to six double bonds.

The dependence of the fatty acid composition of non-ruminants on the nature of the dietary fat (cf. Shorland, 1950, 1952) is further shown by the fact that the perinephric and interscapular fats of the chinchilla rabbit fed on a diet of low fat content stored only 2·0–3·1% of linolenic acid, the main unsaturated fatty acid being oleic, which constituted 31·9–36·7% of the total fatty acids (Clément & Meara, 1951).

Comparative studies on the fatty acid composition of 'glycerides' and phospholipids in mammals (Hilditch & Shorland, 1937; Shorland *et al.* 1952); eggs (Shorland, 1951) and fish (Shorland & Hilditch, 1938; Shorland, 1939; Oliver & Shorland, 1948)

Table 3. *Composition of the C_{18} unsaturated fatty acids of the 'glycerides' and phospholipids from the fatty tissues, liver, kidney and muscle of the rabbit*

Fatty acids are expressed as a percentage (w/w) of the C_{18} unsaturated fatty acids; the figures in parentheses are the percentages (w/w) based on the total fatty acids. M., Male; F., female.

Origin of fat	Conjugated		Non-conjugated		Oleic acid (by diff.)	Triene / Diene
	Diene	Triene	Diene	Triene		
	(*a*) 'Glycerides'					
Fat from abdominal cavity M.	0·9 (0·6)	0·1 (0·1)	13·8 (8·6)	64·3 (40·4)	20·9 (13·1)	4·38
F.	0·1 (0·1)	Trace	16·2 (10·6)	64·2 (42·2)	19·5 (12·8)	3·94
'Yellow fat' Mixed M. & F.	1·2 (0·7)	0·1 (0·1)	13·7 (8·7)	59·3 (37·7)	25·7 (16·3)	3·99
Liver M.	0·2 (0·1)	Trace	36·4 (13·0)	31·0 (11·0)	32·4 (11·5)	0·85
F.	0·7 (0·3)	0·2 (0·1)	33·6 (16·1)	36·5 (17·5)	29·0 (13·9)	1·07
Kidney M.	0·6 (0·3)	0·1 (Trace)	14·4 (7·1)	48·8 (24·1)	36·1 (17·9)	3·26
F.	0·2 (0·1)	0·2 (0·1)	9·2 (5·6)	48·8 (29·9)	41·6 (25·5)	5·21
Longissimus dorsi M.	0·7 (0·3)	0·1 (Trace)	16·0 (8·0)	39·9 (19·8)	43·3 (21·5)	2·40
F.	5·6 (3·1)	0·5 (0·3)	6·7 (3·7)	42·0 (23·1)	45·2 (24·8)	3·45
	(*b*) Phospholipids					
Liver M.	0·5 (0·2)	0·1 (Trace)	62·5 (22·5)	19·0 (6·8)	17·9 (6·4)	0·30
F.	4·3 (1·8)	2·0 (0·8)	54·4 (22·9)	16·1 (6·8)	23·2 (9·7)	0·31
Kidney M.	0·4 (0·2)	0·1 (Trace)	42·1 (16·6)	11·0 (4·3)	46·4 (18·3)	0·26
F.	0·6 (0·3)	Trace	34·4 (14·8)	21·5 (9·2)	43·5 (18·7)	0·62
Longissimus dorsi M.	2·6 (0·9)	0·3 (0·1)	19·9 (6·9)	10·1 (3·5)	67·1 (23·1)	0·46
F.	0·3 (0·1)	Trace	34·8 (12·2)	8·8 (3·1)	56·1 (19·7)	0·25

have indicated that the phospholipids contain more of the higher saturated (C_{18} and above) acids, and of C_{20}–C_{22} unsaturated acids, but less palmitic and C_{16} unsaturated acids. In the present investigations these trends have been generally confirmed, but with certain exceptions. Contrary to expectation, the male and female liver 'glycerides' and the male kidney 'glycerides' contained respectively more of the C_{20}–C_{22} unsaturated fatty acids, and more of the higher saturated (C_{18} and above) fatty acids than the corresponding phospholipids, whereas the male and female longissimus dorsi 'glycerides' were lower in palmitic acid than the corresponding phospholipids. Previous investigations on the 'glycerides' and phospholipids of land mammals have been mainly confined to the liver tissues, and in this respect the rabbit conformed to the general pattern of exhibiting decreased proportions of palmitic acid, but increased amounts of stearic acid in the phospholipids as compared with the 'glycerides'. In the kidney and longissimus dorsi tissues, however, no marked differences in palmitic and stearic acid contents were noted between the 'glycerides' and phospholipids. In accordance with the findings for sheep, oxen, pigs and horses, the C_{20}–C_{22} unsaturated fatty acids were found in substantial amounts in the 'glycerides' of the non-fatty tissues and in trace amounts only in glycerides of the fatty tissues.

In general, the corresponding lipids from the male and female rabbits showed similar fatty acid compositions, particularly in regard to the amount of palmitic acid. In some cases, however, there were differences, such as in the kidney 'glycerides', where the female rabbits showed lower proportions of C_{18}–C_{20} saturated and C_{20}–C_{22} unsaturated acids. In the liver phospholipids there was similarly considerably less C_{20}–C_{22} unsaturated acids in the female rabbit than in the male.

The composition of the C_{18} unsaturated acids shows (see Table 3) that the triene (linolenic) acids concentrate in the 'glycerides' and the diene acids in the phospholipids. This is consistent with the earlier results (Shorland *et al.* 1952) for horse lipids. However, in the rabbit, the ratio C_{18} triene/diene for the glyceride fractions covered a somewhat wider range, from 0·85 to 5·21 as compared with 2·17 to 4·22 found in the horse. Nevertheless, the ratio of C_{18} triene/diene was from 3 to 14 times higher in the glyceride fractions than in the corresponding phospholipid fractions. The glyceride fractions generally incorporated the C_{18} diene and triene acids in much the same ratio as in the diet (C_{18} triene/diene = about 4·0, Shorland *et al.* 1952), but very considerable selectivity was shown by the phospholipids. The present work substantiates the conclusions of Bruce & Shorland (1951) that the selectivity of the phospholipids does not involve merely the selection of higher molecular weight fatty acids, but also the selection of fatty acids based on differences in unsaturation.

SUMMARY

1. The fatty acid composition of the 'glyceride' (non-phospholipid) and phospholipid fractions of the liver, kidney and longissimus dorsi, as well as that of the fatty tissues, of wild rabbits has been determined.

2. In accordance with previous investigations on other animals, the phospholipids generally contained more C_{18} and C_{20} saturated and unsaturated C_{20}–C_{22} fatty acids than the corresponding 'glycerides' but less palmitic and C_{16} unsaturated fatty acids. These differences, however, were not rigid, and exceptions are noted.

3. In agreement with the previous work on the horse lipids, the 'glycerides', as compared with the phospholipids, contained invariably more triene but less diene C_{18} unsaturated acids.

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An Analytical Procedure for the Acid-Soluble Phosphorus Compounds in Rat-Skeletal Muscle

By C. J. THRELFALL

Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

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Few attempts have been made at the identification and estimation of all the phosphorus compounds present in the acid-soluble fraction of skeletal muscle. Recent methods may be divided into two main groups. The first achieves fractionation of the phosphate esters by their precipitation as alkaline-earth salts under conditions of controlled pH and differing ethanol concentrations (LePage, 1948, 1949). Clear-cut separations are not, however, always obtained, and one compound may appear in more than one fraction (Wade & Morgan, 1955). The levels of the esters in each fraction are determined either by the estimation of the carbohydrate moiety or by the increase in inorganic orthophosphate on controlled hydrolysis. Both of these types of estimation are carried out in the presence of similar compounds whose contribution to the final answer is difficult to assess.

Chromatography has provided an alternative approach. Hanes & Isherwood (1949) separated synthetic mixtures by paper chromatography, and Caldwell (1953) utilized their solvent systems for the direct paper chromatography of the acid-soluble extract. Identification of the spots on the chromatograms was made by comparison with known compounds before their estimation by determination of the inorganic phosphate present in the spot after wet ashing. It is difficult, however, to find solvent systems capable of separating adequately the many compounds present, and the detection of trace amounts of esters is only possible when large amounts of extract are applied. This causes overlapping of the smaller spots by compounds present in very much higher concentration, and irregular running of the chromatograms owing to high salt concentrations.

Ion-exchange chromatography has been used widely for separating compounds of a particular class. Cohn & Carter (1950) and Bergkvist & Deutsch (1953, 1954*a*) have successfully separated the nucleotide components but no comprehensive scheme covering all the phosphate esters has been forthcoming.

Wade & Morgan (1955) have elaborated a scheme which appears capable of separating most of the phosphate esters occurring in muscle. This combines paper chromatography with paper electrophoresis but has not yet been applied to biological material. The criticism directed at Caldwell's technique may well apply in this case also. Wade & Morgan point out the difficulty of accommodating the acid-labile phosphates in an analytical scheme involving acid solvents.

Heald (1956) has applied a combination of the fractionation by barium precipitation and the ionophoretic technique of Wade & Morgan (1955) to the analysis of the acid-soluble extract of brain. The difficulties of fractionation into well-defined groups by the barium-precipitation method are apparent, and the separation of the phosphate esters by the ionophoretic method is not complete. Further, some phosphorus compounds remain unidentified and the difficulty of accommodating phosphocreatine in an analytical scheme of this nature is emphasized.

The object of the present method is to overcome these difficulties by first fractionating the phosphate compounds into well-defined and reproducible groups before applying the technique of quantitative paper chromatography.

A preliminary account of this work was given at the Third International Congress of Biochemistry (Brussels, August 1955).

EXPERIMENTAL

Materials

Creatine phosphate (CP) was a synthetic sodium salt obtained from Dr L. A. Stocken, Biochemistry Department, University of Oxford. Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were 'Chromatographically Pure' sodium salts from Schwarz Laboratories Inc., Mount Vernon, U.S.A., and contained traces of adenosine 5-phosphate (AMP) and ADP respectively. Barium 2- and 3-phosphoglycerates and silver barium phosphopyruvate were gifts from Mr R. Hems, Biochemistry Department, University of Oxford. Inosine mono-, di- and tri-phosphates were prepared as barium salts from AMP, ADP and ATP respectively by the method of Klienzeller (1942).

Diphosphopyridine nucleotide (DPN) was prepared from yeast according to Kornberg & Pricer (1953) as described by Aldridge & Cremer (1955). Reduced DPN (DPNH) was prepared according to Lehninger (1952). Triphosphopyridine nucleotide (TPN) was prepared from cow liver by an adaptation of the methods of LePage & Mueller (1949) and Kornberg & Horrecker (1953). Reduced TPN (TPNH)

was prepared in solution by the reduction of TPN with sodium dithionite.

Glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate, ribose 5-phosphate, glucose 1-phosphate and AMP were commercial preparations.

Cations were removed from all phosphate compounds used (except CP) by treatment with Amberlite IR-120 (H). Norit SX 30 Special was a gift from Haller and Phillips Ltd., 14, Wool Exchange, Basinghall Street, London, E.C. 2. Celite 535 was obtained from Johns Manville Co. Ltd., London. *iso*Butanol, *iso*butyric acid and propan-1-ol were B.D.H. Ltd. laboratory reagents. Other reagents were B.D.H. Ltd. A.R. quality.

Methods

Preparation of extracts. Normal muscle samples were obtained as previously described (Stoner & Threlfall, 1954). The muscle sample from the fore-limb was composed mainly of the pectoral muscles and triceps from each side. The hind-limb muscle samples contained the rectus femoris, adductores and gastrocnemius from each side. In both limbs the muscles were rapidly dissected free from fat before freezing in liquid nitrogen. After rapid weighing and the addition of 6 vol. of cold 3% perchloric acid the samples were homogenized in a Nelco 10 blender (MSE, London) for 3 min. and filtered through a Whatman no. 1 filter paper. These operations were carried out at 2°. The blender will homogenize 3–6 g. of muscle satisfactorily; smaller amounts of muscle (1–3 g.) are not dealt with satisfactorily and were ground by hand in a mortar.

Determination of inorganic phosphate (IP) and creatine phosphate. A volume (1 ml.) of the filtered extract was rapidly neutralized with 0·4N-NaOH and diluted to 50 ml. The free and bound creatine were determined on 2·5 ml. samples by the method of Eggleton, Elsden & Gough (1943) as described by Ennor & Rosenberg (1952), with the omission of the *p*-chloromercuribenzoic acid. To obtain consistent results the colour was developed in a water bath at 20° for 30 min.

Creatine phosphate was also determined by the modified method of Berenblum & Chain (1938) as the difference between the IP present in the neutralized extract and the IP present after standing for 25 min. at 20° in the presence of acid molybdate (Ennor & Stocken, 1950), 2·5 ml. samples being used in each case. The procedure was as described below, and 0·9 ml. of 10N-H_2SO_4 was used instead of the ashing mixture.

Phosphorus determinations. Total phosphorus in the initial extract, in the extract after Norit-column treatment and in the pyridine eluate from the Norit column was determined by the method of Fiske & Subbarow (1925). Total phosphorus in the chromatographic spots was determined after ashing the paper (previously treated with acid molybdate mixture as described under chromatography) with 0·5 ml. of sulphuric–perchloric acid mixture (Hanes & Isherwood, 1949); the following modified procedure of Berenblum & Chain (1938) was used. The ashed sample was diluted with 3 ml. of water, cooled and transferred with two washings of 2 ml. of water to a 100 ml. separating funnel containing 2·5 ml. of 2·5% (w/v) ammonium molybdate and 8 ml. of *iso*butanol. The funnels were shaken for 30 sec. on a Microid flask shaker (Baird and Tatlock, London). The organic phase was then shaken twice more with 5 ml. of N-H_2SO_4 for 15 sec., after the lower layer had been run off. The *iso*butanol layer was run into a graduated tube, made up to 10 ml. with ethanol washings from the funnel; reduction was carried out with 0·1 ml. of stannous chloride solution (40%, w/v) as described by Bartley (1953), and readings were made with 1 cm. cells at 725 mμ. on a Unicam SP. 500 spectrophotometer.

Preparation of Norit–Celite columns. Norit SX 30 Special (100 mg.) was mixed with an equal weight of Celite 535 and introduced into a glass column 3–4 mm. in diameter and 8 cm. high sealed with a glass-wool plug at the lower end. To the upper end a wider piece of glass tubing was attached to hold 10 ml. of liquid. The charcoal was packed by tapping the column on the bench and 5 ml. of 8% (v/v) *sec.*-octanol in ethanol forced down the column by compressed-air pressure (4 lb./in.²) followed by 1 ml. of water to remove ethanol. Pretreatment of the Norit–Celite with *sec.*-octanol in ethanol was found to be essential to inactivate the Norit somewhat, otherwise quantitative elution of the nucleotides is not possible with aqueous pyridine.

Preparation of nucleotide fraction. A sample of perchloric acid extract of muscle (5 ml.) was forced down the column followed by 0·5 ml. of water. The adsorbed nucleotides were eluted with 3 ml. of 10% (v/v) aqueous pyridine and this was used, after evaporation to dryness under a current of air and solution in 0·5 ml. of water, for chromatography. Trichloroacetic acid extracts of tissues cannot be used as then the nucleotides are only slowly eluted by aqueous pyridine.

Removal of IP and CP phosphorus. The Norit-treated perchloric acid extracts from several columns were combined and transferred to a 20 ml. separating funnel; the IP and CP phosphorus were removed by the addition of 10% (w/v) ammonium molybdate and 0·5 vol. of pentanol. The two phases were allowed to stand for 25 min. at room temperature with occasional shaking. After a final vigorous shake the organic layer was removed and the aqueous phase shaken twice more with pentanol to remove traces of phosphomolybdic acid complex. The volume of ammonium molybdate solution used was determined empirically as the least necessary to remove the IP and the IP formed during the catalytic breakdown of the CP as determined initially. This was found to be 0·4 ml. of 10% (w/v) ammonium molybdate for 500 μg. of P in 5 ml. of extract. Separate experiments had shown that perchloric acid could replace H_2SO_4 in the breakdown of CP catalysed by acid molybdate.

Excess of molybdate was removed by passing a vigorous current of H_2S through the cold solution for 20 min., to precipitate the molybdate as molybdenum sulphide, and allowing to stand overnight at 2°. After filtration at 2° through Whatman no. 42 filter paper the solution was aerated to remove H_2S.

The removal of IP and CP phosphorus was found to be essential to obtain good chromatograms of the sugar phosphates, since the presence of IP in high concentrations tended to obscure the other constituents and cause uneven running of the chromatograms.

Preparation of extract for chromatography. The remaining phosphates were precipitated from the ice-cold extract, after aeration, by the addition of finely divided baryta until the solution was markedly alkaline to phenolphthalein, 4 vol. of ethanol was then added (Sacks, 1949) and the mixture allowed to stand at −15° overnight. The barium salts were centrifuged at −5° and washed once with cold

ethanol. Water (5 ml.) was added to the precipitate, followed by Amberlite cation exchanger IR-120(H) to remove barium. The aqueous phase was pipetted off and the resin washed twice with 2 ml. of water. The combined washings were adjusted to 10 ml., and after addition of more dry resin were shaken and filtered through Whatman no. 42 filter paper. After neutralization with N-NH_3 soln. a measured volume was freeze-dried in a 25 ml. round-bottomed flask. The small amount of white solid obtained was dissolved in 0·2 ml. of water for chromatography.

Chromatography. Chromatography was carried out on Whatman no. 1 papers (50 cm. × 23 cm.) treated with ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952) at 25° in an all-glass apparatus. Application of material to the paper was made with an Agla micrometer syringe (Burroughs Wellcome and Co., London); successive spots (5 μl.) were dried with a stream of cold air from a hair-drier.

The concentrated pyridine eluate from the Norit–Celite columns were applied and developed in the *iso*butyric acid–ammonia solvent of Krebs & Hems (1952) for 20 hr. Good separations of inosine monophosphate, ATP, ADP and AMP were obtained. Guanosine and uridine triphosphates which are present in trace amounts (Bergkvist & Deutsch, 1953, 1954*b*, *c*) were not separated from each other but could be separated in the ammonium sulphate–propan-2-ol solvent system of Bergkvist & Deutsch (1953). Nucleotide spots were located by viewing under ultraviolet light (Hanovia Chromatolite) and the absence of other spots containing non-nucleotide phosphorus confirmed by the molybdate-spraying technique of Hanes & Isherwood (1949).

The solution of the freeze-dried intermediates (50–100 μl. corresponding to 0·25–0·5 g. of muscle) was chromatographed for 16 hr. in the *tert.*-butanol–picric acid solvent of Hanes & Isherwood (1949), in an upward direction along the minor axis of the paper; the two short sides of the paper were secured together with cellulose self-adhesive tape (Sellotape) to form a cylinder. Inside the chromatographic tank H_2S was generated in a beaker containing barium sulphide and H_2SO_4. After drying at room temperature the paper was developed in a downward direction for 48 hr. along its major axis in the propanol–ammonia system of Hanes & Isherwood (1949). Phosphorus-containing spots were located by the acid molybdate-spraying technique as described above and estimated as previously described.

Compounds were identified by co-chromatography with known markers.

RESULTS

Results presented by Ennor & Rosenberg (1952) suggest that the phosphocreatine content of tissues determined by the two methods employed here does not always coincide. Data presented in Table 1 suggest that in rat-skeletal muscle, at least, good agreement is obtained by the two methods, indicating that no other phosphate compounds labile to acid molybdate are present.

The efficiency of the Norit–Celite columns for the retention of nucleotides is shown in Table 2 and the recovery with 10% aqueous pyridine of the adsorbed nucleotide is seen to be quantitative. Table 3 shows that non-nucleotide phosphate esters are not retained by the columns but are recovered quantitatively in perchloric acid medium after passing down the column.

Table 4 shows that inorganic phosphate can be removed from the phosphorylated glycolytic intermediates by the molybdate–pentanol technique, leaving the organically bound phosphorus (except for CP) in the aqueous phase in quantitative yield.

Table 1. *Estimation of creatine phosphate*

Creatine phosphate (6·2 μmoles) was dissolved in 1 ml. of cold 3% perchloric acid; 0·5 ml. was rapidly neutralized with NaOH and diluted to 25 ml. Portions (2·5 ml.) were taken for estimations of IP and CP phosphorus and for free and total creatine. Muscle was extracted with 6 vol. of cold 3% perchloric acid, filtered, neutralized and diluted (1:50). Portions (2·5 ml.) were taken as for synthetic CP. Each pair of muscle values is from a separate animal.

	Phosphate	Creatine
Synthetic CP (μmoles/ml.)	6·16	6·30
Muscle CP (μmoles/g.)	15·6	14·9
	17·0	16·7
	17·3	17·5
	15·85	15·7
	16·7	16·5
	13·8	13·6
	16·5	16·3
	15·7	15·9
Mean ± standard error of mean	16·05 ± 0·35	15·9 ± 0·42

Table 2. *Adsorption and recovery of nucleotides from Norit columns*

Nucleotides were dissolved in 5 ml. of 3% perchloric acid and passed down a Norit column (100 mg.); elution was with 3 ml. of 10% (v/v) aqueous pyridine.

Nucleotide applied to column (μmoles)		Ultraviolet absorption of perchloric acid effluent at 250–270 mμ.	Nucleotide recovered in pyridine eluate (μmoles)
ATP	5·70	+ + +	4·0
	4·10	+	3·9
	2·87	–	2·74
	0·40	–	0·40
	0·18	–	0·18
	0·09	–	0·09
ADP	0·53	–	0·52
AMP	4·80	–	4·90
Inosine monophosphate	4·30	+ + +	2·60
	0·48	–	0·48
Uridine triphosphate	0·78	+ + +	0·59
	0·35	+	0·33
	0·22	–	0·22
DPN	1·41	–	1·42
	4·70	–	4·69
DPNH	4·18	–	4·29
TPN	4·62	–	4·75
TPNH	4·80	–	4·75

Recoveries of some typical phosphate esters during the barium-precipitation stage are shown in Table 5. Glycogen, which is stated (LePage, 1949) to interfere with the precipitation of these compounds, is adsorbed on the Norit and is not eluted with 10% aqueous pyridine.

Table 6 shows the distribution of the total phosphorus in the acid-soluble fraction of rat-skeletal muscle into its major fractions together with the distribution of metabolites within these fractions. The figures contain the values from the fore- and hind-limb muscle of each animal, the only significant difference being in the IP and CP fraction. The fore limb has a mean IP of 9·47 μmoles/g. and a mean CP of 16·33 μmoles/g., and the hind limb has values of 11·33 and 14·98 μmoles/g. for IP and CP respectively. This is doubtless because the fore limb was always sampled first, the CP in the hind limb probably breaking down to a greater extent *in situ* during the removal procedure.

For the estimation of DPNH the muscle samples were extracted with perchloric acid at room temperature to convert DPNH into a compound which is stable towards acid. Otherwise DPNH is slowly transformed during the chromatographic separation of the nucleotides in the *iso*butyric acid solvent and gives rise to two spots with trailing between them. The procedure is otherwise unaltered. It was found that elevated IP (13·6 μmoles/g.) and lowered CP (11·34 μmoles/g.) values were obtained for normal muscle by this modification. The values for the nucleotides were little altered by running the chromatograms at right angles in the acetone–water system of Rafter, Chaykin & Krebs (1954) or in the propanol–ammonia system used for the sugar phosphate separations, except that in the latter system the DPN is completely destroyed as would be expected from its known lability in alkaline media (Schlenk, Euler, Heiwinkel, Gleim & Nyström, 1937). The acetone–water system has been shown to separate ATP from TPN and DPNH which have the same R_F values in the *iso*butyric acid solvent system; the results indicate that TPN and DPNH are not present in any appreciable quantity in normal resting muscle.

Contrary to expectation a spot corresponding to either 2- or 3-phosphoglycerate was not found with the *tert.*-butanol–picric acid/propanol–ammonia solvent systems, although a consistently occurring unknown spot was found, second only in quantitative significance in the glycolytic intermediate fraction. Synthetic 2- or 3-phosphoglycerates added to a perchloric acid extract of muscle before manipulation did not produce a spot coincident with this unknown spot.

Glucose- and fructose-6-phosphate were not sufficiently well separated to allow their separate estimation, but with a longer run in the picric acid–*tert.*-butanol solvent system this could probably be achieved.

A spot corresponding to the position occupied by synthetic phosphopyruvic acid was not found with the *tert.*-butanol–picric acid/propanol–ammonia solvent systems.

Table 3. *Recovery of non-nucleotide phosphates in perchloric acid after passing through Norit columns*

Compounds were dissolved in 5 ml. of 3% perchloric acid and passed through the column.

	μmoles of P/5 ml. of perchloric acid	
	Before column	After column
Orthophosphate	0·72	0·72
Pyrophosphate	0·77	0·77
Creatine phosphate	0·50	0·50
Glucose 1-phosphate	0·72	0·72
Glucose 6-phosphate	0·53	0·51
Fructose 6-phosphate	0·23	0·23
Fructose diphosphate	0·39	0·37
β-Glycerophosphate	0·76	0·75
Barium 3-phosphoglycerate	0·55	0·58

Table 4. *Recovery of glycolytic intermediates after removal of IP*

Compounds were dissolved in 3% perchloric acid and 0·4 ml. of 10% ammonium molybdate (w/v) and 0·5 vol. of pentanol were added. The two phases were shaken in a separating funnel and the aqueous phase was re-extracted twice with pentanol. Excess of molybdate was removed with H_2S.

	μmoles of P/5 ml. of perchloric acid			
	Before treatment		After treatment	
Compound	Inorganic P	Organic P	Inorganic P	Organic P
Fructose 6-phosphate	8·1	1·34	0·00	1·36
Glucose 6-phosphate	8·1	1·35	0·01	1·40
Glucose 1-phosphate	8·1	1·50	0·01	1·50
Fructose diphosphate	8·1	1·50	0·00	1·46
Barium 3-phosphoglycerate	8·1	1·82	0·03	1·84
β-Glycerophosphate	8·1	1·55	0·01	1·60

DISCUSSION

The results presented indicate that 95% of the phosphorus present in a perchloric acid extract of rat-skeletal muscle can be consistently allocated among the known constituents. It is essential to estimate the CP separately in a scheme involving acid-extraction procedure and solvent systems because of its lability under these conditions.

The charcoal-column technique is based on observations of Crane & Lipmann (1953) and Fiske (1934), who showed that adenine nucleotides could be separated from IP and the glycolytic intermediates by shaking with Norit charcoal. This has proved successful in obtaining a clear-cut separation of the nucleotides present in the perchloric acid extract from the other phosphate compounds. No substances absorbing in the region 250–270 mμ. are present in the perchloric acid extract after Norit treatment, nor are non-nucleotide phosphate esters adsorbed by the Norit. It is seen in Table 2 that 100 mg. of Norit columns will retain 4–5 μmoles of nucleotides containing the adenine moiety, but that substitution of the amino group of adenine by the hydroxyl group, as in inosine monophosphate, decreases considerably the amount retained, and that uridine triphosphate, containing no amino group but two hydroxyl groups on a pyrimidine moiety, is only retained to a small extent by the Norit columns. This technique has been successfully applied to liver extracts by increasing the amounts of Norit and Celite by 50%, keeping the diameter of the column the same and by using 5 ml. of a similar extract. The increased amount of Norit was made necessary by the higher concentrations of hydroxylated nucleotides found in liver.

The absence of AMP in muscle agrees with the recent findings of Mommaerts (1955) and indicates that in resting muscle all the adenine mononucleotide occurs as either the di- or tri-phosphate.

The absence of a spot corresponding to phosphoglycerate and the appearance of another spot not co-chromatographed with any of the glycolytic intermediates used is not readily explained, but Caldwell (1953) has shown that in the propanol–ammonia system phosphoglycerate has an R_F value of 1·7 relative to IP, which compares well with that found for the unknown spot. Also, LePage (1948) has reported the presence of phosphoglycerate in

Table 5. *Precipitation of phosphate esters with baryta*

The compounds were dissolved in 10 ml. of 3% perchloric acid. Solid baryta was added until the solution was pink to phenolphthalein and followed by 4 vol. of ethanol. The solution was set aside for 24 hr. at −15°, centrifuged at −5°, treated with IR-120 (H) and adjusted to 10 ml.

	μmoles of P/10 ml. of perchloric acid	
	Before treatment	After treatment
β-Glycerophosphate	0·82	0·86
Fructose diphosphate	0·94	0·90
Glucose 6-phosphate	1·21	1·24
Barium 3-phosphoglycerate	1·90	1·80
Fructose 1-phosphate	1·13	1·12
Glucose 1-phosphate	1·32	1·34
Mixture of above compounds	3·48	3·48

Table 6. *Distribution of phosphorus compounds in perchloric acid extract of rat-skeletal muscle*

Values (μmoles of P/g. wet wt.) are means ± standard error of mean. Number of observations in each case is shown in parentheses.

	Determined constituent	Calculated totals	Determined total
(1) Determined immediately			
IP	10·4 ±0·35 (20)	25·9	—
CP	15·5 ±0·29 (20)		
(2) Pyridine eluate			
ATP	18·52±0·26 (20)		
ADP	2·10±0·45 (20)	22·28	22·0±0·40 (14)
DPN	1·14±0·03 (20)		
Guanosine and uridine triphosphates	0·52±0·03 (20)		
(3) Perchloric acid extract after Norit–Celite column			
Fructose diphosphate	0·45±0·03 (19)		
Glucose 6-phosphate + fructose 6-phosphate	1·83±0·15 (20)		
Glucose 1-phosphate	0·38±0·04 (11)	29·26	30·0±0·43 (15)
Phosphoglycerate*	0·70±0·14 (20)		
IP + CP	25·9±0·45 (20)		
(4) Total phosphorus	53·6±0·71 (15)	51·54	52·0
		by addition of (2) and (3)	

* Not positively identified: see text.

Table 7. *Comparison of results obtained with those of LePage* (1948)

	Present results (μmoles/g. wet wt.)	Results of LePage (μmoles/g. wet wt.)
Total acid-soluble P	53·6	50·7
IP	10·4	7·48
CP	15·48	16·30
ATP	6·17	5·42
ADP	1·05	0·59
AMP	0	1·55
DPN	0·59	0·17
Guanosine and uridine triphosphates	0·47	0
Glucose 6-phosphate + fructose 6-phosphate	1·83	2·83
Glucose 1-phosphate	0·38	0·80
Fructose diphosphate	0·23	0·14
Phosphoglycerate*	0·70	1·40
Ribose 5-phosphate	0	0·22

* See text.

rat-skeletal muscle, and it seems possible therefore that this unknown spot is phosphoglycerate and that the compounds used may not have been the naturally occurring acids.

Comparison of results found here with those of LePage (1948) is made in Table 7. There is good agreement between the two differing methods. LePage's technique cannot differentiate between the various nucleotide di- and tri-phosphates or between inosine monophosphate and AMP, both of which can occur in muscles under pathological conditions. LePage gives concentrations of AMP and ribose 5-phosphate which would easily have been detected by the present method if present. In normal muscle the amount of nucleotides other than the adenine nucleotides is small, but in other organs such as liver this is not so; for analysis in such cases the procedure outlined is superior.

SUMMARY

1. A method is presented for the quantitative estimation of 95% of the phosphate compounds present in a perchloric acid extract of tissues.

2. The nucleotide components are adsorbed on Norit charcoal and eluted with aqueous pyridine before their separation by paper chromatography.

3. After removal of the inorganic phosphorus and creatine phosphate-phosphorus as the phosphomolybdic acid complex, the glycolytic intermediates are precipitated as barium salts. After removal of barium with cation-exchange resin the solutions are lyophilized and the compounds separated by paper chromatography.

4. Results are presented showing the application of this technique to rat-skeletal muscle.

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αε-Diaminopimelic Acid Metabolism and Sporulation in *Bacillus sphaericus*

By JOAN F. POWELL and R. E. STRANGE
Microbiological Research Establishment (Ministry of Supply), Porton, Wiltshire

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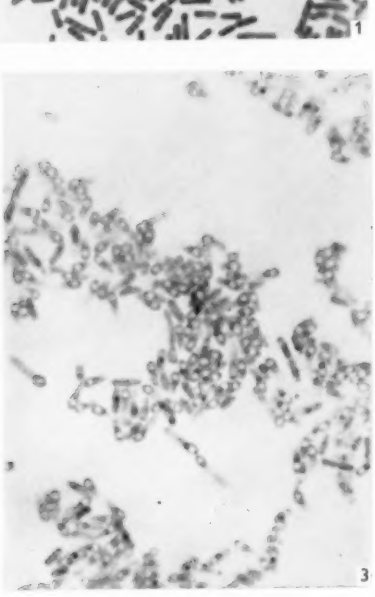
The metabolism of αε-diaminopimelic acid (DAP) by sporulating cells is of interest on two counts. First, by analogy with the conversion of lysine into piperidine-2-carboxylic acid (Rothstein & Miller, 1953; Lowy, 1953) it seems possible that DAP may be the precursor of pyridine-2:6-dicarboxylic acid (dipicolinic acid). This compound is built up as a calcium salt in the sporulating cell, finally accounting for 10–15 % of the dry weight of the resting spore (Powell & Strange, 1953; Powell, 1953; Powell & Strange, 1956). Secondly, DAP is a constituent of the 'spore peptide', a soluble non-dialysable complex containing DAP, glutamic acid, alanine and hexosamines (Strange & Powell, 1954). This peptide is a component of the spore coat from which it is freed during germination, probably by the activation of an intracellular lytic system similar to lysozyme (Powell & Strange, 1956; Strange & Dark, 1956, 1957). A peptide of the same or similar constitution appears to be widely distributed in bacterial cell walls (Cummins & Harris, 1956; Salton, 1956; Strange & Dark, 1957). We were unable to find a correlation between DAP content and ability to sporulate in *Bacillus subtilis*, *Bacillus megatherium*, *Bacillus anthracis* and *Bacillus pumilus*: all the cultures we examined contained 1–2 % (w/w) of DAP (Powell, Strange & Hunter, unpublished results). *Bacillus sphaericus* proved, however, to be an interesting exception. In an asporogenous strain of this organism (Powell & Hunter, 1955) we could detect no DAP in 30 mg. dry weight of cells. In the parent sporogenous strain vegetative cells contained no DAP, whereas sporulating cells and spores contained normal amounts. This report includes some observations on the general biochemical changes, in particular the build-up of DAP, which occur during sporulation of *B. sphaericus* and two varieties of the species.

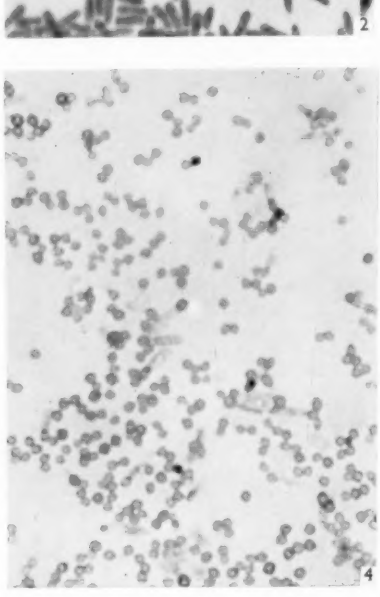
ORGANISMS AND METHODS

B. sphaericus, *National Collection of Type Cultures* (*N.C.T.C.*) 9602. This strain, when tested according to the scheme of Knight & Proom (1950), appeared to be a typical *B. sphaericus*. On the same basis, *B. sphaericus* N.C.T.C. 7582 was classified as var. *fusiformis* and a laboratory strain of *B. sphaericus* isolated by Dr J. L. Peel as var. *rotans*. The asporogenous strain was derived from *B. sphaericus* N.C.T.C. 7582 by repeated subculture as previously described (Powell & Hunter, 1955). Growth and sporulation of *B. sphaericus* N.C.T.C. 9602 was studied in potato-extract medium (Robinow, 1951) enriched with 1/10 vol. of a solution containing acid and trypsin-hydrolysed casein, yeast extract, glycerophosphate and glutamine (CCY; Gladstone & Fildes, 1940). Growth and sporulation of the fusiformis variety was studied in this medium and also in a meat extract–peptone medium (Tarr, 1933). Vegetative cells and spores of the rotans variety were grown in both these media and also in a 10 % yeast-extract medium (Barker & Beck, 1942). Cultures were grown at 37° in shaken 1 l. flasks containing 200 ml. of medium inoculated with 10^5 spores/ml. Sporulation of all three organisms was usually complete after 24 hr. growth in the potato extract–CCY and meat extract–peptone media. The rotans variety sporulated completely after 48 hr. growth in the yeast-extract medium, but sporulation of the other two organisms was very poor, i.e. less than 10 %. Cells were harvested at various stages of growth and sporulation, washed once with 0·9 % NaCl, then suspended in water and freeze-dried. The method of preparation of soluble and insoluble fractions from these freeze-dried cells after mechanical disintegration (Mickle, 1948) and the determination of their DAP and hexosamine content has been described in detail in an earlier paper (Powell & Strange, 1956). The ultraviolet-absorption spectra of cell extracts and the calcium content of whole cells were also determined as previously described (Powell & Strange, 1956).

Assay of DAP decarboxylase

Cells of the fusiformis variety were harvested at various stages of growth and sporulation in the potato extract–CCY medium, washed twice with water and freeze-dried overnight. They were tested the following day. Gross changes in cell structure and permeability occur during sporulation: we therefore used disintegrated freeze-dried cells in these enzyme tests. Cell suspensions (5–7·5 ml.) containing 10–20 mg. dry wt. of cells/ml. in 0·1 M sodium potassium phosphate, pH 6·9, and one drop of tributyl citrate, were first cooled to 0° then again at 10 min. intervals during disintegration. This usually took 45–60 min. In these early experiments with the fusiformis variety, the semi-quantitative method of DAP decarboxylase assay described by Hoare & Work (1955) was used. The disintegrated cell suspension (1 ml.) was incubated at 37° with 0·2 ml. of 2 mM synthetic DAP and 0·2 ml. of pyridoxal phosphate solution (50 μg./ml.). The reaction mixture (0·2 ml.) was added to 0·4 ml. of ethanol after 0, 1, 2 and 4 hr. incubation and centrifuged. The protein-free supernatant (0·1 ml.) was spotted on to Whatman no. 1 paper together with standard amounts of DAP, lysine and cadaverine for comparison, and





Cells of *B. sphaericus* N.C.T.C. 9602 (× 1500) from cultures of varying ages in potato extract–CCY medium. Heat-fixed films stained with methylene blue: (1) 6 hr. culture: featureless vegetative cells; (2) 9 hr. culture: first signs of sporulation; appearance of volutin granules; (3) 15 hr. culture: most cells contain a well-developed spore and a volutin granule; (4) 24 hr. culture: mainly free spores.

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run for 18 hr. in the solvent system of Rhuland, Work, Denman & Hoare (1955). The DAP used in these tests was a commercial preparation which appeared to contain mainly the *meso* isomer (see below).

In later experiments with *B. sphaericus* N.C.T.C. 9602, the DAP decarboxylase activities of freshly reaped disintegrated cells and of intact and disintegrated freeze-dried cells were compared. In these tests it was found that the decarboxylase activity of disintegrated cells was increased by adding mM thiolacetic acid (Dewey, Hoare & Work, 1954) to the suspension before disintegration and by substituting octan-2-ol for tributyl citrate as the antifoaming agent. In these experiments with *B. sphaericus* N.C.T.C. 9602, the manometric method of decarboxylase assay was used in an atmosphere of N_2 (Dewey *et al.* 1954). The flasks contained 20–40 mg. (dry wt.) of cells suspended in 2 ml. of 0·1 M sodium potassium phosphate, pH 6·9, and 0·1 ml. of pyridoxal phosphate solution (100 μg./ml.). The side arms contained 0·5 ml. of 25 mM *meso*-DAP and 0·1 ml. of M citric acid. $Q^{N_2}_{CO_2}$ values (μl. of CO_2/mg. dry wt of cells/hr.) were calculated from the initial steady rate of CO_2 production. In most experiments the end products of the reaction were examined by paper chromatography (Antia, Hoare & Work, 1957) after protein precipitation with 2 vol. of ethanol.

Assay of DAP racemase

Freeze-dried cells of *B. sphaericus* N.C.T.C. 9602 were suspended in 0·1 M borate buffer, pH 8·5, containing mM thiolacetic acid and octan-2-ol. The cell suspensions (20 mg. dry wt. of cells/ml.) were disintegrated, and 0·8 ml. volumes incubated at 37° with 0·4 ml. of 10 mM *meso*-DAP or with 0·4 ml. of 10 mM LL-DAP. Samples (0·2 ml.) of the reaction mixture were taken after 0, 1 and 18 hr. incubation and examined on a paper chromatogram after deproteinizing with 0·4 ml. of ethanol (Antia *et al.* 1957).

Chemicals. The synthetic DAP used in the early DAP decarboxylase assays was a commercial preparation supplied by L. Light and Co. Examination on paper chromatograms indicated that it contained approximately 10% of the LL isomer. Pure *meso*- and LL-DAP were very kindly provided by Dr E. Work. Calcium dipicolinate was isolated from germination exudates of *B. megatherium* (Powell, 1953). Piperidine-2:6-dicarboxylic acid was obtained by the reduction of dipicolinic acid (Andersson & Soine, 1950) which was prepared by the oxidation of 2:6-lutidine (Hess & Wissing, 1915). Pyridoxal phosphate was a commercial preparation supplied by Roche Products Ltd.

RESULTS

Whole cells (5 mg.) of the fusiformis variety reaped after 8, 12, 16 and 20 hr. growth in potato extract–CCY medium were examined for their DAP content. No DAP could be detected in the 8 and 12 hr. cultures, whereas the 16 and 20 hr. cultures contained approximately 0·5% of DAP. Similarly, in a series of cultures grown in meat extract–peptone medium, cells reaped after 7 and 10 hr. growth contained no DAP, whereas 15, 20 and 24 hr. cultures contained 0·5–1%. In both these media, the first signs of sporulation appeared after 12–14 hr. growth. At this stage, most cells contained one and occasionally two volutin granules (Powell & Hunter, 1955), and many cells appeared 'tadpole' shaped in heat-fixed stained films (cf. Plate 1, 2). In 15–17 hr. cultures, sporulation was proceeding strongly, and most cells contained a well-defined developing spore as well as a volutin granule (cf. Plate 1, 3). At 24 hr. the cultures in meat extract–peptone consisted almost entirely of free spores. Although growth was heavier in the potato extract–CCY medium, sporulation was incomplete, i.e. 70–80%.

The hexosamine and DAP content of the soluble and insoluble fractions of vegetative and sporulating cells of the fusiformis variety are given in Table 1. In this series is included a 48 hr. sporulated culture washed six times with water to free it as far as possible from vegetative material. During sporulation, which began after about 16 hr. growth, there was a sudden decrease in the hexosamine content of the insoluble fraction of the cell and a corresponding increase in the soluble fraction. This change was most marked in the 48 hr. washed spore suspension. No DAP could be detected in hydrolysates of either

Table 1. *Distribution of hexosamine and DAP in cells of* B. sphaericus *at various stages of growth and sporulation in potato extract–CCY medium*

Organism	Age (hr.)	Appearance of culture	Hexosamine (g./100 g. dry wt.)			DAP (g./100 g. dry wt.)	
			Whole cells	Insoluble fraction	Soluble fraction	Insoluble fraction	Soluble fraction
B. sphaericus	8	Vegetative	—	12·1	1·2	0	0
var. *fusiformis*	10	Vegetative	—	15·6	1·1	0	0
N.C.T.C. 7582	12·5	Vegetative	4·2	12·1	1·4	0	0
	15	First signs of sporulation	—	14·1	1·7	0	0
	17·5	Sporulating strongly	—	8·7	2·3	Faint traces	Faint traces
	20	70–80% free spores	—	6·5	3·8	Faint traces	Faint traces
	48	Washed spore suspension	3·4	1·4	4·6	0	0·5–1·0
B. sphaericus	6	Vegetative	3·3	9·6	1·6	0	0
N.C.T.C. 9602	7·5	Vegetative	—	9·1	1·8	0	0
	9	First signs of sporulation	—	10·6	1·7	0	0
	15	Sporulating strongly	—	5·5	4·6	Traces	0·5–1·0
	22	>95% free spores	6·4	2·1	7·8	Faint traces	1·0–2·0

fraction of the 8, 10, 12·5 and 15 hr. cultures. In the 17·5 and 20 hr. cultures, traces of DAP were detected in both fractions, whereas in the 48 hr. spore suspension the insoluble fraction contained no DAP and the soluble fraction 0·5–1 %.

Similar results were obtained with *B. sphaericus* N.C.T.C. 9602 in potato extract–CCY and in meat extract–peptone medium: no DAP could be detected in vegetative cells, whereas spores contained 1–2 %. Sporulation was more rapid than in the fusiformis variety, 'tadpole' shaped granular cells appearing after 8–9 hr. growth (Plate 1, 2). At 15 hr. (Plate 1, 3) sporulation was practically complete and lysis of the sporangia had begun. At 24 hr. (Plate, 1, 4) the culture consisted almost entirely of clean free spores with very little vegetative debris. The DAP and hexosamine contents of the soluble and insoluble cell fractions are included in Table 1. It was very difficult to assess the DAP content of the insoluble fraction of the 22 hr. culture

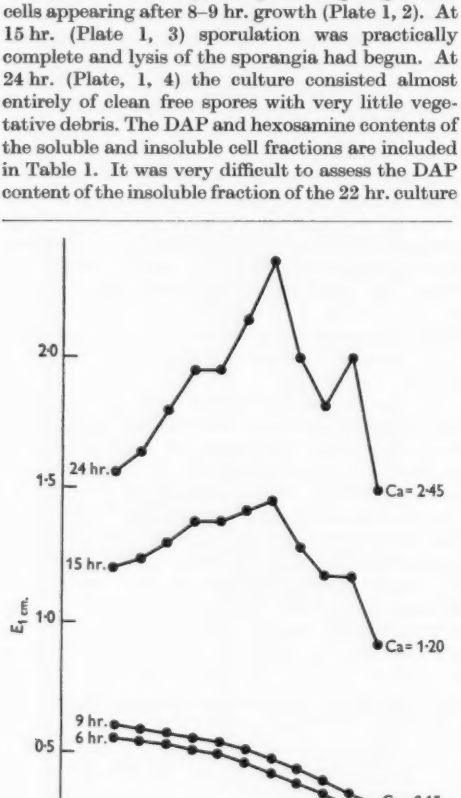

Fig. 1. Ultraviolet-absorption spectra of extracts from cells of *B. sphaericus* N.C.T.C. 9602 at various stages of growth and sporulation in potato extract–CCY medium. Extracts contained the equivalent of 1 mg. (dry wt.) of cells/ml. Light path 1 cm. Calcium contents of whole cells are given as g./100 g. (dry wt.) of cells.

owing to the presence of an unidentified constituent occupying the same position on the chromatogram as DAP. This unidentified constituent gave a blue colour with ninhydrin, whereas DAP gave an olive-green colour. Probably because sporulation and lysis of the sporangia was more nearly complete in *B. sphaericus* N.C.T.C. 9602 than in the fusiformis variety, the change in distribution of hexosamine and the appearance of DAP in the soluble fraction of the cell at sporulation was more striking. Similarly, the appearance of the characteristic ultraviolet-absorption spectrum of calcium dipicolinate in cell extracts, and the build-up of calcium in the cell during sporulation, was more marked in *B. sphaericus* N.C.T.C. 9602 (Fig. 1).

The DAP content of vegetative cells and spores of the rotans variety grown in potato extract–CCY, meat extract–peptone and yeast-extract media was also determined. Again, we could not detect DAP in vegetative cells, whereas spores contained about 1 %. Vegetative cells of *B. sphaericus* N.C.T.C. 9602 and of the fusiformis variety grown in yeast-extract medium also appeared to contain no DAP. Both these strains sporulated very poorly in yeast extract.

The above results suggested to us the possibility that vegetative cells of *B. sphaericus* might contain (*a*) an analogue of the DAP spore peptide in which DAP was replaced by lysine, and (*b*) a DAP decarboxylase system which either disappeared or was inhibited during sporulation. Evidence in support of these suggestions is now presented.

Isolation of non-dialysable peptides containing hexosamine from vegetative cells and spores of B. sphaericus

Non-dialysable peptides were isolated from germination exudates and from spore extracts as previously described by Strange & Powell (1954). We first examined the peptide which appeared in germination exudates from spores of the fusiformis variety. Approximately 80–90 % germination occurred when spores which had been preheated at 60° for 2 hr. were incubated for 1 hr. in 4 mM L-alanine and 20 mM sodium potassium phosphate, pH 7·3 (cf. Powell, 1950). Under these conditions 500 ml. of a suspension containing 2 mg. dry wt. of spores/ml. yielded 21 mg. of peptide. This peptide contained 30 % of hexosamine, and its main constituents were DAP, glutamic acid, alanine, aspartic acid, glucosamine and the substituted hexosamine (SH) for which a structure has recently been suggested by Strange (1956). Most of the common amino acids were present in small amounts and these still persisted after repeated treatment of aqueous solutions of the peptide preparation with chloroform and amyl alcohol (Sevag, 1934). Some purification was effected by paper electrophoresis

and elution of the major component with reference to a paper stained with naphthalene black (cf. Strange & Powell, 1954). This eluted material contained DAP, glutamic acid, alanine, aspartic acid, glucosamine and SH with only faint traces of glycine, serine, valine and leucine. A preparation of nondialysable peptide (5·6 mg.) from disintegrated resting spores (235 mg.) was also examined. This contained 29 % of hexosamine, and its constituents were DAP, glutamic acid, alanine, glucosamine and SH, with faint traces of glycine and serine. Germination exudate peptide from *B. sphaericus* N.C.T.C. 9602 was not examined. A preparation of extract peptide from these spores contained DAP, glutamic acid, alanine, and the two hexosamines together with small amounts of aspartic acid. Its hexosamine content was 35 %.

It was recently shown (Powell & Strange, 1956; Strange & Dark, 1957) that extracts from disintegrated spores of *B. cereus* attacked vegetative cell-wall preparations of this organism and of *B. megatherium* and *B. subtilis*. A non-dialysable peptide containing hexosamine was liberated, similar in composition to that isolated from spore extracts and germination exudates (Strange & Powell, 1954). A similar lytic system was present in extracts from spores of a non-virulent strain of *B. anthracis*, and was also associated with the insoluble fraction of disintegrated *B. megatherium* spores. In an attempt to isolate hexosamine-containing peptides from vegetative cells of *B. sphaericus*, cell-wall preparations from 6 and 8 hr. cultures of *B. sphaericus* N.C.T.C. 9602 were incubated with the soluble and insoluble fractions from disintegrated spores of this organism. The test system (1 ml.) contained 5 mg. of the cell-wall preparation previously heated at 100° for 15 min. (Strange & Dark, 1957) and 10 mg. of the freeze-dried soluble or insoluble fraction from disintegrated spores, suspended in McIlvaine buffer, pH 7·4, and 0·2 mM-$CoSO_4$. No liberation of hexosamine-containing material could be detected after incubation for 2 hr. at 60°, or after continued incubation for 18 hr. at 37°. In a similar test system, extracts from *B. cereus* spores did not attack vegetative cell walls of *B. sphaericus*, nor did extracts from *B. sphaericus* spores attack vegetative cell walls of *B. cereus*. Similar negative results were obtained with spores of the fusiformis variety.

Vegetative cell-wall preparations of the fusiformis variety were next treated with lysozyme. This enzyme has been shown to attack vegetative cell walls of *Bacillus* and other species, liberating a number of dialysable and non-dialysable components (Salton, 1953, 1956). The washed, insoluble fraction (80 mg.) from a 10 hr. vegetative culture of the fusiformis variety (560 mg.) was suspended in 10 ml. of 0·4 % NaCl containing 2 mg. of crystalline egg-white lysozyme. Visible lysis occurred during incubation for 2 hr. at 37° on a rotary shaker. No lysis was apparent in a control suspension incubated without lysozyme. The suspensions were centrifuged and the supernatant was removed and cooled to 0°. Protein was precipitated with 3 % (w/v) trichloroacetic acid and the protein-free supernatant was neutralized with solid $NaHCO_3$. After treatment with chloroform and amyl alcohol (Sevag, 1934) the solution was dialysed and freeze-dried; 16 mg. of white solid was obtained. This material was hydrolysed for 3 hr. at 100° in 6 N-HCl and examined on two-dimensional paper chromatograms. It contained lysine, glutamic acid, alanine, aspartic acid, glucosamine and SH. Its hexosamine content was 39 %. There was also present in these hydrolysates an unidentified constituent giving a blue colour with ninhydrin and having the same R_F value as arginine in phenol–ammonia and as glycine in collidine–lutidine. It gave no Sakaguchi reaction on the paper (Acher & Crocker, 1952) and no significant colour with *p*-dimethylaminobenzaldehyde. When the time of hydrolysis of the cell-wall component was prolonged to 20 hr. there was a marked increase in intensity of the lysine and aspartic acid spots but no significant decrease of the unidentified component.

A non-dialysable peptide of very similar constitution containing 36 % of hexosamine was obtained from cell-wall preparations of *B. sphaericus* N.C.T.C. 9602 by incubation with lysozyme. Hydrolysates of this material also gave an unidentified spot on paper chromatograms, which occupied a corresponding position to that of the unidentified constituent from the fusiformis variety.

Cell-wall preparations from a 10 hr. vegetative culture of *B. megatherium* in potato extract–CCY medium were also treated with lysozyme. These cell walls contained approximately 5 % (w/w) of DAP. A soluble non-dialysable peptide was isolated from the reaction products. This peptide contained DAP, glutamic acid, alanine, glucosamine and SH. Its hexosamine content was 24 %.

A summary of the experiments with *B. sphaericus* is given in Table 2. There was a small and variable amount of phosphorus present in all the peptide preparations examined. The ultraviolet absorption of a peptide preparation (2·2 mg./ml.) from vegetative cells of *B. sphaericus* N.C.T.C. 9602 indicated that its phosphorus content of 0·32 % was not due to a nucleic acid component. The peptide preparations also contained a variable amount of carbohydrate, excluding hexosamine. This was usually less than 3 %. The peptides liberated by lysozyme gave a positive reaction for *N*-acetylhexosamine (Aminoff, Morgan & Watkins, 1952).

Table 2. *Amino acids and amino sugars in peptides from* B. sphaericus

Organism	Source of peptide	Hydrolysis time (hr.)	DAP	Aspartic acid	Glutamic acid	Alanine	Lysine	Unidentified substance	Hexosamine	SH*	Total amino sugars as glucosamine after hydrolysis for 2 hr. (g./100 g.)	Morgan–Elson reaction† as *N*-acetylglucosamine (g./100 g.)
B. sphaericus var. *fusiformis* N.C.T.C. 7582	8 hr. vegetative cells	16	0	+	+ +	+ +	+	+	+ +	+	32	3·4
	10 hr. vegetative cells‡	16	0	+	+ +	+ +	+	+	+ +	+	39	—
	Spore extract	16	+	0	+ +	+ +	0	0	+ +	+ +	29	0
	Spore exudate	16	+	+	+ +	+ +	0	0	+ +		30	0
B. sphaericus N.C.T.C. 9602	6 hr. vegetative cells‡	3	0	+	+ +	+ +	±	+	+ +	+ +	35	2·0
		20	0	+	+ +	+ +	±	+	+ +	+ +		
	9 hr. vegetative cells‡	3	0	+	+ +	+ +	±	+	+ +	+ +	37	—
		20	0	+	+ +	+ +	±	+	+ +	+ +		
	Spore extract	16	+	±	+ +	+ +	0	0	+ +	+ +	35	0

* Unidentified hexosamine.
† Peptide heated for 7·5 min. with dilute alkali before addition of Ehrlich reagent.
‡ Peptide obtained by the action of lysozyme on the insoluble fraction of disintegrated cells.

DAP decarboxylase in B. sphaericus

Early semi-quantitative experiments with freeze-dried disintegrated cells of the fusiformis variety indicated that vegetative cells contained a very active DAP decarboxylase system. Lysine appeared as a reaction product, but no cadaverine could be detected. The cells therefore contained no active lysine decarboxylase. Decarboxylation of DAP proceeded more rapidly in the presence of added pyridoxal phosphate. The results of a typical assay are given in Table 3. The activity of sporulating cells was considerably lower than that of vegetative cells, whereas spores showed relatively little decarboxylase activity. The lower activity of spores and sporulating cells might be due to a reduced enzyme content or to the presence of inhibitory substances which are not present in vegetative cells. Calcium dipicolinate was tested as a possible inhibitor of DAP decarboxylase. The addition of 1·5 mg. of this substance/ml. to a suspension of disintegrated vegetative cells containing 10 mg. dry wt. of cells/ml. did not reduce its decarboxylase activity. Calcium dipicolinate is excreted during spore germination (Powell, 1953). We therefore compared the activity of washed germinated spores, obtained as described above, with that of resting spores. In this experiment, the final volume of the germinated-spore suspension was adjusted to allow for the 30% decrease in cell dry wt. which occurs during germination (Powell & Strange, 1953). There was no apparent increase in DAP decarboxylase activity during germination.

The DAP decarboxylase activity of cells of *B. sphaericus* N.C.T.C. 9602 was next determined, by the more accurate manometric method of assay, and with pure *meso*-DAP as substrate. It was first established that under the conditions of assay (see Methods) the enzyme was saturated with substrate and with pyridoxal phosphate. The initial steady rate of CO_2 production was proportional to the dry wt. of cell material present, and the total volume of CO_2 produced during the reaction indicated that

Table 3. *Rough assay of DAP decarboxylase activity of freeze-dried disintegrated cells of* B. sphaericus *N.C.T.C.* 7582 *var.* fusiformis

Cells (10 mg. dry wt.) were present in 1 ml. of 0·1M sodium potassium phosphate, pH 6·9, with 0·2 ml. of 25 mM synthetic DAP and 0·2 ml. of pyridoxal phosphate solution (50 μg./ml.), and were incubated at 37°. Results are expressed as percentage decarboxylation of DAP.

Age (hr.)	Appearance of cells	Time of incubation (hr.) 1	2	4
10	Vegetative	50–75	90	90
15	Sporulating	25	75	80–90
24	70–80% free spores	10–20	10–20	20

Table 4. *DAP decarboxylase activity ($Q_{CO_2}^{N_2}$) of vegetative cells, sporulating cells and spores of* B. sphaericus *N.C.T.C. 9602*

Age (hr.)	Appearance of culture	Storage at −15° in freeze-dried state (days)	$Q_{CO_2}^{N_2}$ Intact cells	$Q_{CO_2}^{N_2}$ Disintegrated cells
7	Featureless vegetative cells	0*	—	24·0
		1	30·0	—
		2	28·2	21·6
		3	27·3	18·6
9	Vegetative cells showing some granule formation	1	27·0	—
		2	19·8	18·0
9	Vegetative cells showing some granule formation	0*	—	21·9
		3	21·0	17·7
		4	20·7	—
15	Sporulating: i.e. most cells containing a well-defined spore	0*	—	12·0
		1	4·2	—
		2	3·0	9·5
15	Sporulating	0*	—	8·6
		1	3·0	8·4
		4	2·8	12·0
15	Sporulating	0*	—	14·4
		1	3·2	12·6
		2	3·0	11·2
24	Washed spore suspension	0*	1·4	4·5
		0*	2·4	6·4
		0*	1·1	2·7

* Freshly reaped cells tested before freeze-drying.

only one carboxyl group of DAP was attacked. Decarboxylation in N_2 by both intact and disintegrated cells proceeded at least twice as rapidly in the presence of added pyridoxal phosphate.

Freeze-dried intact vegetative cells of *B. sphaericus* N.C.T.C. 9602 gave $Q_{CO_2}^{N_2}$ values between 20 and 30. Disintegrated freeze-dried cells and also disintegrated freshly reaped cells gave rather lower $Q_{CO_2}^{N_2}$ values (18–24). There was a slight loss of activity when freeze-dried cells were stored at −15° for several days. Intact freeze-dried sporulating cells, i.e. cells containing a well-defined developing spore, gave relatively low $Q_{CO_2}^{N_2}$ values (3–4), but here disintegration produced a three- or four-fold increase in decarboxylase activity: thus $Q_{CO_2}^{N_2}$ values of 9–14 were obtained with disintegrated freeze-dried sporulating cells and also with disintegrated freshly reaped cells. Spore suspensions gave very low $Q_{CO_2}^{N_2}$ values (1–2·5) when tested intact, and higher values (3–6) after disintegration. These results are shown in Table 4.

Several authors have reported instances of enzymic activity shown by spore extracts but not by intact spores (Murrell, 1952; Powell & Hunter, 1956; Church, 1955). In these instances the enzyme was heat-stable in the intact spore but became heat-labile when the spore was disintegrated. Powell & Hunter (1956) in a study of the heat-stable adenosine deaminase of *B. cereus* spores found that this enzyme also became heat-labile during germination. We therefore compared the heat stability of DAP decarboxylase in vegetative cells, sporulating cells, resting spores and germinated spores of *B. sphaericus* N.C.T.C. 9602. Suspensions of intact vegetative cells, sporulating cells and resting spores in 0·1 M sodium potassium phosphate, pH 6·9, were heated at 60° for 15 min. Their DAP decarboxylase activities were measured before and after disintegration and compared with those of intact and disintegrated unheated cells. The results obtained are shown in Table 5. Vegetative cells tested intact and disintegrated retained very little or no decarboxylase activity after heat treatment. Most of the activity shown by intact sporulating cells and spores was lost during heat treatment, but these heated cells still showed markedly increased decarboxylase activity when disintegrated. This effect was most noticeable with spores which, when disintegrated after heat treatment, showed 50–100 % of the activity of the unheated disintegrated suspension. On the other hand, when disintegrated spore suspensions were heated at 60° for 15 min., their DAP decarboxylase activity was completely lost. There was little change in decarboxylase activity during spore germination, whether this took place in 4 mM L-alanine (see above) or in the potato extract–CCY medium supplemented with 4 mM L-alanine. $Q_{CO_2}^{N_2}$ values for washed disintegrated germinated spores obtained in these two media were 5·7 and 4·7. Approximately 90 % of this

Table 5. *Heat stability of DAP decarboxylase in vegetative cells, sporulating cells and spores of* B. sphaericus *N.C.T.C.* 9602

Age (hr.)	Appearance of culture	Unheated cells $Q_{CO_2}^{N_2}$		Cells heated at 60° for 15 min. $Q_{CO_2}^{N_2}$	
		Intact	Disintegrated	Intact	Disintegrated
7	Featureless vegetative cells	28·2	21·6	0	0
9	Vegetative cells showing some granule formation	18·0	15·0	0	0·8
15	Sporulating strongly	3·2	12·6	0·6	3·0
15	Sporulating strongly	3·0	8·4	0·6	3·2
24	Free spores	1·4	4·5	0	4·5
24	Free spores	2·4	6·4	0·2	4·0
24	Free spores	1·1	2·7	—	1·05

activity was lost when the germinated spores were heated at 60° for 15 min. before disintegration.

To determine whether there was a rapid increase in DAP decarboxylase activity during the development of the germinated spore into a vegetative cell, preheated resting spores (2 mg. dry wt. of spores/ml.) were shaken at 37° in the potato extract–CCY medium supplemented with 4 mM L-alanine. Stained films made at 30 min. intervals indicated that, after incubation for 2 hr., at least 90 % of the spores had developed into vegetative cells. The suspension was centrifuged and the cells were resuspended in 0·1 M sodium potassium phosphate, pH 6·9, to give 15 mg. dry wt. of cells/ml. Their DAP decarboxylase activity was determined after disintegration. A $Q_{CO_2}^{N_2}$ value of 5·0 was obtained.

The above experiments with germinated spores of *B. sphaericus* N.C.T.C. 9602 and the early experiments with the fusiformis variety indicated that the low DAP decarboxylase activity of disintegrated resting spores was probably not due to their calcium dipicolinate content. This conclusion was supported by further experiments with *B. sphaericus* N.C.T.C. 9602. The rate of decarboxylation of DAP by disintegrated vegetative cells was unaffected by the addition of equimolar amounts of calcium or sodium dipicolinate. These compounds were not themselves decarboxylated. Piperidine-2:6-dicarboxylic acid was also tested under the same conditions as a possible inhibitor of DAP decarboxylation. Added in equimolar amounts it was not inhibitory and, tested alone, it was not decarboxylated.

The DAP decarboxylase activity of vegetative cells from a 9 hr. culture of the fusiformis variety in potato extract–CCY medium was compared with that of a 9 hr. culture of the asporogenous strain in the same medium. Freeze-dried intact cells of the sporogenous strain gave a $Q_{CO_2}^{N_2}$ value of 18·0, compared with 7·9 for the asporogenous strain. In another pair of 9 hr. cultures in the same medium, $Q_{CO_2}^{N_2}$ values for freshly reaped disintegrated cells of the sporogenous and asporogenous strains were 13·5 and 8·1. The corresponding values for intact freeze-dried cells from these cultures were 15·3 and 8·4. In a single experiment with the rotans variety, a $Q_{CO_2}^{N_2}$ value of 23·5 was obtained with intact freeze-dried vegetative cells from a 18 hr. culture in yeast-extract medium.

Under the conditions of assay, vegetative cells, sporulating cells and spores of *B. sphaericus* N.C.T.C. 9602 showed no DAP racemase activity. The *meso* isomer of DAP was completely decarboxylated to lysine by vegetative cells during 1 hr. incubation, and by sporulating cells and spores during overnight incubation. There was no detectable conversion of the LL into the *meso* isomer, and no lysine formation was apparent with the LL isomer.

DISCUSSION

B. sphaericus appears to be atypical of the *Bacillus* genus (Antia *et al.* 1957) in its high DAP decarboxylase activity and the absence of DAP from its cells during vegetative growth. DAP was present in spores of *B. sphaericus* and, as in other *Bacillus* species (Strange & Powell, 1954), appeared in spore extracts and germination exudates as a constituent of a non-dialysable peptide containing DAP, alanine, glutamic acid and hexosamines. The germination exudate peptide from the fusiformis variety also contained aspartic acid, and traces of aspartic acid were present in the extract peptide from *B. sphaericus* N.C.T.C. 9602. On previous evidence (Strange & Dark, 1956, 1957) it at first seemed likely that these peptides were derived from the spore coat. Extracts from spores and sporulating cells of several *Bacillus* species have been shown to contain a lytic, lysozyme-like system which attacked vegetative cell-wall and spore-coat preparations, releasing peptides of very similar constitution to those just described (Powell & Strange, 1956; Strange & Dark, 1957). It was suggested that this lytic system might be concerned in the lysis of the sporangium and freeing of the spore during sporulation, and also in the initiation of the early stages of the germination process. In the present study, although we isolated similar peptides from spore extracts and germination

exudates of *B. sphaericus*, we failed to demonstrate a lytic system in these spores. We have shown, however, that vegetative cell-wall preparations of *B. sphaericus* were attacked by lysozyme and that a peptide was released containing lysine, aspartic acid, alanine, glutamic acid and hexosamines but no DAP. It is possible that, if present, the lytic system of *B. sphaericus* spores may be relatively feeble. This suggestion is supported by the observation that spores of *B. sphaericus* germinate relatively slowly even after prolonged preheating. It is also possible that the lytic system of *B. sphaericus* spores has greater specificity than that of the other *Bacillus* species examined and that it can only liberate the peptide containing DAP from its particular linkages in the *B. sphaericus* spore. Another possibility, rather less likely in our opinion, is that in *B. sphaericus*, the DAP–hexosamine peptide is not a component of the coat of the intact spore and that it appears in the soluble fraction of the disintegrated spore independently of a lytic system. The peptides released from vegetative cell-wall preparations by lysozyme all contained an unidentified constituent reacting with ninhydrin. The position of this material on paper chromatograms was similar to that of a constituent of cell-wall hydrolysates of certain lactobacilli described by Cummins & Harris (1956). These authors found that, on prolonged hydrolysis, the intensity of the aspartic acid and lysine spots increased while that of the unidentified constituent decreased. They therefore considered the unidentified constituent to be a peptide relatively resistant to acid hydrolysis and composed chiefly of aspartic acid and lysine. Although on prolonged hydrolysis of the vegetative cell-wall peptides from *B. sphaericus* the intensity of the aspartic acid and lysine spots increased, we could not detect any diminution of the unidentified constituent. Guex-Holzer & Tomcsik (1956) have described a constituent of hydrolysed mucopolysaccharide from a *Bacillus* species occupying a similar position on phenol/collidine–lutidine chromatograms to that of the unidentified material from *B. sphaericus*, but giving a positive reaction with *p*-dimethylaminobenzaldehyde. We hope to isolate the unidentified constituent from *B. sphaericus* for further investigation.

Vegetative cells of *B. sphaericus* contained a very active DAP decarboxylase system requiring pyridoxal phosphate as a cofactor. Sporulation was accompanied by the appearance of DAP as a cell constituent and by diminished DAP decarboxylase activity. Although the DAP decarboxylase activity of spores was relatively low, the activity of sporulating cells was still approximately 50% that of vegetative cells, i.e. there was no sudden disappearance of DAP decarboxylase activity during the onset of sporulation, although at this stage DAP appeared as a cell constituent. These observations were made with disintegrated cells saturated with pyridoxal phosphate. It is possible that in the living cell, DAP decarboxylase activity might be greatly reduced by a shortage of pyridoxal phosphate, and that the onset of sporulation is associated with a disturbance of pyridoxal phosphate synthesis. The possibility will be investigated. In this connexion, it was interesting to find that an asporogenous strain of *B. sphaericus* derived from the fusiformis variety showed roughly only one-half of the DAP decarboxylase activity of the parent strain. No trace of DAP could be detected in the asporogenous strain.

Freeze-dried intact sporulating cells and spores appeared to be less permeable to DAP than were vegetative cells: the DAP decarboxylase activity of sporulating cells and spores was increased approximately threefold by disintegration. Several examples of the relative inactivity of intact compared with disintegrated spores in enzyme tests have already been given (Murrell, 1952; Church, 1955). The DAP decarboxylase activity of *B. sphaericus* spores is a further example. As in previous instances (Murrell, 1952; Church, 1955; Powell & Hunter, 1956) the DAP decarboxylase of *B. sphaericus* spores was heat-stable in the intact spore, but became heat-labile during disintegration or germination. A possible explanation for this behaviour was discussed in a previous study of the adenosine deaminase system of *B. cereus* spores (Powell & Hunter, 1956).

In sporulating cells of *B. sphaericus*, presumably both the sporangium and the developing spore contained DAP decarboxylase. It seems likely that the decarboxylase activity which survived heat treatment was associated with the developing spore. On the other hand, the difference in decarboxylase activity between intact and disintegrated sporulating cells was considerably greater than the heat-stable activity of the cell (Table 5). This would imply either that (*a*) the developing spore contained both heat-stable and heat-labile DAP decarboxylase activity, or that (*b*) the wall of the sporangium itself had become less permeable to DAP. At the moment we have no evidence in favour of either of these possibilities.

The DAP decarboxylase system of disintegrated *B. sphaericus* cells appeared to be less sensitive to inhibition by dipicolinic acid than was the partially purified system from *Aerobacter aerogenes* studied by Dewey *et al.* (1954). This difference might be attributed to the presence of relatively large amounts of inert protein in our system, or to a difference in properties between the enzymes from *B. sphaericus* and *Aero. aerogenes*. It appears that, in *B. sphaericus*, the accumulation of calcium

dipicolinate in the sporulating cell was not responsible for its diminished DAP decarboxylase activity. Germinated spores and 'first-generation' vegetative cells also showed relatively low activity. It seems that the actual amount of enzyme present in the cell decreased to a minimum in the spore and increased to a maximum during the period of rapid vegetative growth.

SUMMARY

1. In *Bacillus sphaericus* and in *Bacillus sphaericus* var. *fusiformis* and *Bacillus sphaericus* var. *rotans* αε-diaminopimelic acid (DAP) was present in sporulating cells and spores but not in vegetative cells.

2. As in other *Bacillus* species, pyridine-2:6-dicarboxylic acid was built up as a calcium salt in the developing spore.

3. A peptide containing DAP, aspartic acid, glutamic acid, alanine and hexosamines appeared in spore extracts and germination exudates. A peptide containing lysine, aspartic acid, glutamic acid, alanine and hexosamines was isolated from vegetative cell walls by treatment with lysozyme.

4. Vegetative cells showed strong DAP decarboxylase activity, stimulated by pyridoxal phosphate; $Q_{CO_2}^{N_2}$ values in nitrogen were 20–30. Lysine was a reaction product but no cadaverine was detected. Sporulating cells were less active ($Q_{CO_2}^{N_2}$ 10–15). Spores showed relatively little activity ($Q_{CO_2}^{N_2}$ 3–6). These values were obtained with disintegrated cells. Sporulating cells and spores gave lower $Q_{CO_2}^{N_2}$ values when tested intact.

5. The DAP decarboxylase activity of spores was relatively heat-stable. There was no increase in decarboxylase activity during germination and development to the first vegetative cell. During spore germination, the decarboxylase system became less heat-stable.

6. Pyridine-2:6-dicarboxylic acid and piperidine-2:6-dicarboxylic acid were not decarboxylated by vegetative cells of *B. sphaericus*; in equimolar concentration, they did not inhibit the decarboxylation of DAP by these cells.

7. Vegetative cells, sporulating cells and spores showed negligible DAP racemase activity.

8. Some possible relationships between DAP metabolism and sporulation in *B. sphaericus* are discussed.

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Some Observations on the Role of Copper Ions in the Reduction of Phosphomolybdate by Ascorbic Acid and their Application in the Determination of Inorganic Orthophosphate

By J. L. PEEL
Agricultural Research Council Unit for Microbiology, The University, Sheffield, 10

and B. C. LOUGHMAN*
Botany School, University of Cambridge

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The reduction of molybdate in the presence of phosphate gives rise to a blue compound, and under suitable conditions the colour intensity is proportional to the amount of orthophosphate present. This principle forms the basis of several methods which have been described for the determination of phosphate and a variety of reducing agents has been used for this purpose. In the method of Lowry & Lopez (1946) ascorbic acid is used at pH 4, in contrast to the strongly acid conditions used in most other methods, and in addition a relatively low molybdate concentration is employed. This reduces the breakdown of labile phosphates such as creatine phosphate, acetyl phosphate and ribose 1-phosphate, which is otherwise very rapid (Fiske & Subbarow, 1929; Lipmann & Tuttle, 1944; Lowry & Lopez, 1946), to such an extent that it can be corrected for. This method thus permits the determination of inorganic phosphate in the presence of labile phosphate esters.

Difficulty was experienced in applying the method as described by Lowry & Lopez to the determination of phosphate in reaction mixtures containing extracts of a rumen micro-organism. Similar observations were made in the presence of heat-coagulated potato extracts, and Lowry & Lopez (1946) observed a slight interference with concentrated preparations of liver and brain extracts. The results presented in this paper indicate that the reduction of phosphomolybdate by ascorbic acid requires copper ions and show that the process is powerfully inhibited by metal-binding agents; a suitable modification to the method as described by Lowry & Lopez is suggested. A preliminary account of part of this work has already been given (Peel, Fox & Elsden, 1955).

Since this work was completed, Bruemmer & O'Dell (1956) have reported that phosphomolybdate reduction is inhibited by the reduced, but not the oxidized, forms of glutathione and cysteine, and that copper sulphate reverses these inhibitions and also that due to liver extract. Their results, which are in agreement with our own, are referred to in more detail below.

* Present address: Department of Agriculture, University of Oxford.

MATERIALS AND METHODS

Bacterial extract. This was prepared from the rumen organism LC1 described by Elsden, Volcani, Gilchrist & Lewis (1956). The organism was grown on a medium containing 1·4% (w/v) of sodium lactate and 1% (v/v) of corn-steep liquor; growth conditions and the preparation of extracts of vacuum-dried cells were essentially the same as those described by Stadtman & Barker (1949) for *Clostridium kluyveri*. The full details will be given in a separate paper and need only be considered briefly. The main factor of interest to the present investigation is that the dried cells were extracted with 0·01 M potassium phosphate buffer, pH 6·8, with the addition of 0·03% $Na_2S,9H_2O$ to maintain anaerobic conditions. The clear supernatant after centrifuging will be referred to as 'bacterial extract'. For certain experiments the extract was deproteinized by the addition of 7 ml. of the ammonium sulphate–acetate reagent of Lowry & Lopez (1946) for each millilitre of bacterial extract. The supernatant obtained after centrifuging will be referred to as 'deproteinized bacterial extract'.

Liver and brain extracts. The two tissues were provided by Dr R. Whittam from a freshly killed guinea pig and samples were homogenized with 0·125 M sodium acetate buffer, pH 4, in the apparatus of Potter & Elvehjem (1936), made of stainless steel. With liver, 10 g. of tissue was homogenized with 40 ml. of buffer for 4 min.; with brain, 4 g. of tissue was homogenized with 20 ml. of buffer for 1 min. After removal of the coarser debris by centrifuging at 1800 *g* for 10 min., the supernatant was deproteinized by saturating with $(NH_4)_2SO_4$ and recentrifuging at 9000 *g* for 20 min. The supernatants, which will be referred to as 'deproteinized extracts', were stored at 2° and used within a few days.

Chemical reagents. All inorganic chemicals except Cu_2O were of A.R. grade. Cuprous oxide and ascorbic acid were obtained from J. Preston Ltd. (Sheffield), cysteine hydrochloride from Light and Co. Ltd. and the ethylenediaminetetraacetic acid and sodium diethyldithiocarbamate were of laboratory reagent grade obtained from British Drug Houses Ltd. and Hopkin and Williams Ltd. respectively. Lithium acetyl phosphate was prepared with the help of

Dr G. C. N. Jayasuriya from *iso*propenyl acetate by the method of Stadtman & Lipmann (1950). This preparation had been stored for a considerable time and was contaminated with inorganic phosphate. Solutions were assayed by the method of Lipmann & Tuttle (1945).

Cuprous chloride reagent. Cuprous oxide (0·5 g.) was dissolved in approx. 10 ml. of hot N-HCl and the solution cooled to precipitate the Cu_2Cl_2. The precipitate was filtered off, washed with a little water and then used to saturate a sample of 0·125 M sodium acetate buffer, pH 4.

Reduction of phosphomolybdate. Formation of the blue chromogen by the reduction of phosphomolybdate was studied under the conditions described by Lowry & Lopez (1946). Phosphate, as KH_2PO_4, was contained in 0·5 ml. or less, and to this were added other substances as indicated, followed by 0·125 M sodium acetate buffer, pH 4, to a total volume of 5 ml., and 0·5 ml. of 1% (w/v) ascorbic acid. The reaction was started by adding 0·5 ml. of 1% (w/v) ammonium molybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] in 0·05 N-H_2SO_4. The course of the development of the blue colour was followed with the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with the red filter (no. 205) supplied with the instrument. Colour intensities were measured relative to a blank cell containing water and are expressed in terms of the scale readings of this instrument (range 0–100 scale units) which are directly proportional to optical density.

When phosphate determinations were carried out on incubation mixtures, these were first deproteinized with ammonium sulphate–acetate reagent in the same way as the bacterial extract described above. Where acetyl phosphate was present, colour intensity was measured 10 and 20 min. after addition of the molybdate reagent and the intensity at zero time, obtained by extrapolation, taken as the measure of true inorganic phosphate (cf. Lowry & Lopez, 1946). The rate of colour development was found to vary with different batches of reagents and even by as much as 25% with the same reagents on different days (cf. the various experiments of Table 1). All operations were carried out at uncontrolled room temperature (approx. 20°) and temperature variations may therefore account for this latter effect, since Lowry & Lopez (1946) observed that at 35° colour development was complete in one-third of the time required at 25°, and we have observed an approximate doubling of the rate of colour development between 15° and 25°.

RESULTS

Inhibition of phosphomolybdate reduction by bacterial extract. The present detailed investigations arose after attempts to determine inorganic phosphate in an incubation mixture resulting from the action of an extract of the rumen organism LC 1 on pyruvate (Peel, 1956). It was known that a considerable amount of inorganic phosphate was present, and colour development during the assay was expected to be completed within 5 min. After 10 min., however, no measurable colour had developed, though a marked colour did appear after 30 min. It was concluded that some component of the incubation mixture was interfering with colour development and the trouble was traced to the bacterial extract

The effect of a quantity of deproteinized bacterial extract, equivalent to that present in the assay on the incubation mixture, is shown in Fig. 1. In the absence of interfering substances (curve *A*), colour development was linear with time and with 20 μg. of P reached a maximum value in 4–8 min. Thereafter only a very slow change, an increase of approx. 1 scale unit/hr., was observed. In the presence of bacterial extract the rate of colour development remained linear with time but was considerably depressed, and the reaction was not complete after 10 min. (curve *B*, Fig. 1). Similar effects have been observed with heat-coagulated extracts of potato, and by Lowry & Lopez (1946) in the presence of relatively high concentrations of liver and brain extracts, though the bacterial extract was a far more potent inhibitor than these other preparations.

Inhibition by sulphide. By comparison with other extracts, the most unusual feature of the bacterial extract appeared to be the presence of 0·03% of sodium sulphide. The effect of a quantity of sulphide equal to that originally present in the bacterial extract tested was therefore examined and complete inhibition of colour development observed (curve *C*, Fig. 1). Further experiments showed that the inhibition was complete with a sulphide concentration as low as 5×10^{-7} M (Expt. 1, Table 1),

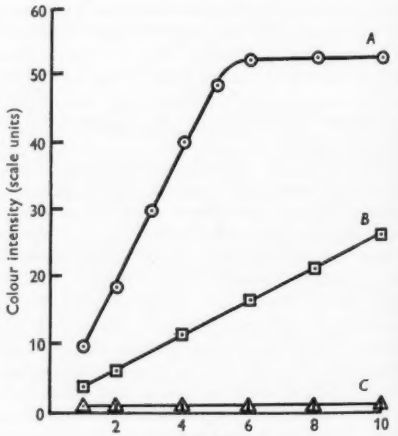

Fig. 1. Effect of deproteinized bacterial extract and of sodium sulphide upon phosphomolybdate reduction. Tubes contained: 0·5 ml. of KH_2PO_4 containing 20 μg. of P, 0·5 ml. of deproteinized bacterial extract or $Na_2S.9H_2O$ to give the concentrations indicated, 0·5 ml. of 1% ascorbic acid; 0·5 ml. of 1% ammonium molybdate in 0·05 N-H_2SO_4 was added at zero time and 0·125 M sodium acetate buffer, pH 4, to make total vol. 6 ml. Curve *A*, no additions; curve *B*, 0·83% of deproteinized bacterial extract present; curve *C*, $1·3 \times 10^{-6}$ M-Na_2S present.

Table 1. *Inhibition of phosphomolybdate reduction by metal-binding agents*

Tubes contained 20 μg. of P and 1 ml. of inhibitor; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml. Rates were measured over the linear phase of colour development. The four experiments were done on different days and two different batches of reagents used, one for Expts. 1 and 4 and the other for Expts. 2 and 3.

Expt.	Inhibitor	Concn. of inhibitor (M)	Rate of colour development (scale units/min.)	Percentage inhibition
1	Sodium sulphide	0	9·4	—
		5×10^{-9}	9·3	1
		5×10^{-8}	5·5	41
		5×10^{-7}	0	100
		5×10^{-6}	0·3	97
2	Diethyldithiocarbamate	0	11·0	—
		1×10^{-7}	9·3	15
		2×10^{-7}	8·0	27
		5×10^{-7}	1·3	88
		5×10^{-6}	0·45	96
3	Ethylenediaminetetraacetic acid	0	13·0	—
		5×10^{-7}	9·2	29
		2×10^{-6}	7·3	44
		5×10^{-6}	1·4	89
		5×10^{-5}	0·6	95
4	Cysteine	0	7·3	—
		$1{\cdot}7 \times 10^{-5}$	7·3	0
		$1{\cdot}7 \times 10^{-4}$	2·3	68
		$1{\cdot}7 \times 10^{-3}$	0·95	87

corresponding to 3 μm-moles of sulphide in the mixture. This amount was far less than the amount of phosphate present (645 μm-moles) and therefore suggested that the sulphide interferes with some catalytic process involved in phosphomolybdate reduction.

Inhibition by other metal-binding agents. It was suspected that sulphide might inhibit by binding a metal, and this was confirmed when other metal-binding agents were tested (Table 1). Diethyldithiocarbamate inhibited the rate of colour development almost as effectively as sodium sulphide, and ethylenediaminetetraacetic acid was only slightly less inhibitory, 5×10^{-6} M giving an almost complete inhibition. Cysteine, although a much less powerful complexing agent, nevertheless gave a strong inhibition at concentrations of the order of 10^{-3} M.

Bruemmer & O'Dell (1956) observed a marked inhibition with 8×10^{-5} M cysteine, in agreement with our own results. These authors also noted a comparable effect with glutathione and showed that the oxidized forms of these two compounds did not inhibit. This latter observation is consistent with the idea of inhibition by metal-binding since the free sulphydryl group is known to be important in metal-binding (Albert, 1952).

Effect of added cupric ions on colour development during phosphomolybdate reduction. The oxidation of ascorbic acid by air, both enzymically and non-enzymically, is stimulated by copper ions (see Meiklejohn & Stewart, 1941) and ascorbic acid oxidase is in fact a copper protein (Dawson & Mallette, 1945). It therefore appeared possible that copper ions might be involved in the reduction of phosphomolybdate by ascorbic acid, and their effect upon the system was investigated. The findings were rather complex, three distinct effects being observed as the concentration of cupric ions was increased.

Table 2. *Effect of low concentrations of added copper ions on colour development during phosphomolybdate reduction*

Tubes contained 20 μg. of P and 1 ml. of $CuSO_4$ to give the concentrations indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml. Rates were measured as in Table 1.

Concn. of added $CuSO_4$ (M)	Rate of colour development (scale units/min.)	Colour intensity after 10 min. (scale units)
0	10	50
2×10^{-8}	14	50
2×10^{-7}	19	50
2×10^{-6}	35	50
2×10^{-5}	170	55

Copper sulphate up to 2×10^{-5} M caused a marked increase in the initial linear rate of colour development (Table 2). Otherwise the general course of the reaction was unaffected (cf. curve *A*, Fig. 1 and

curve *A*, Fig. 2). After the initial rapid increase there was no further change with time and the final colour intensity attained after 10 min. was not affected by $CuSO_4$ up to 2×10^{-6} M. With 2×10^{-5} M-$CuSO_4$, the first signs of a second effect began to appear, namely an enhancement of the final colour.

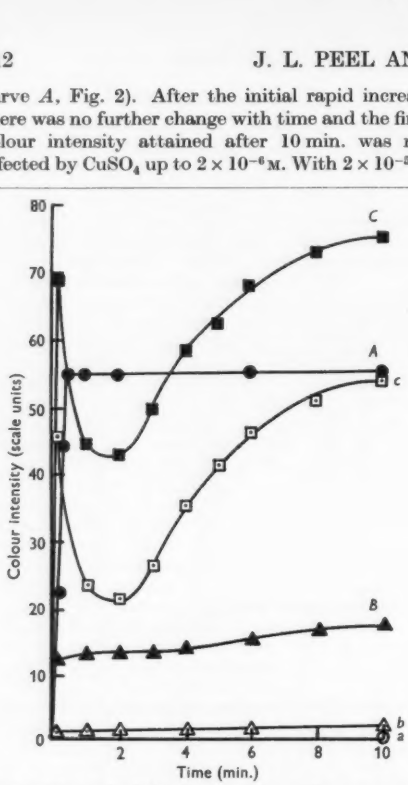

Fig. 2. Effect of added copper ions on the progress of colour development during phosphomolybdate reduction. Tubes contained: 0·5 ml. of KH_2PO_4 and 1 ml. of $CuSO_4$ to give the amounts indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1, total vol. 6 ml. Curve *A*, 2×10^{-5} M-$CuSO_4$ and 20 μg. of P; curve *B*, $1{\cdot}7 \times 10^{-4}$ M-$CuSO_4$ and 4 μg. of P; curve *C*, $1{\cdot}7 \times 10^{-3}$ M-$CuSO_4$ and 4 μg. of P; curves *a*, *b*, *c*, as for *A*, *B*, *C* respectively but no P present.

In studying the effect of higher concentrations of $CuSO_4$, the amount of phosphate present was reduced to one-fifth of that used in the preceding experiments in order to keep the colour intensities produced within the range of the colorimeter. With concentrations of $1{\cdot}7 \times 10^{-4}$ M and above, there is a marked increase in the colour intensity after 10 min. (Table 3; also compare slopes, Table 4). With $1{\cdot}7 \times 10^{-4}$ M-$CuSO_4$, the initial rapid appearance of colour was followed by a slow increase which still persisted after 10 min. (curve *B*, Fig. 2). At this concentration of $CuSO_4$ the enhanced colour obtained after 10 min. was directly proportional to the amount of phosphate present over the range 0–20 μg. of P.

With still higher concentrations of $CuSO_4$ a third effect became apparent, namely, the development of a pronounced colour in the absence of phosphate. Without phosphate, the colour after 10 min. was

Table 3. *Effect of higher concentrations of added copper ions on colour development during phosphomolybdate reduction*

Tubes contained 4 μg. of P and 0·01 M-$CuSO_4$ to give the concentrations indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1; total vol. 6 ml.

Concn. of added $CuSO_4$ (M)	Colour intensity after 10 min. (scale units): 4 μg. of P present	Colour intensity after 10 min. (scale units): No P present
0	11	0
$1{\cdot}7 \times 10^{-4}$	17	2
$3{\cdot}3 \times 10^{-4}$	23	3
$6{\cdot}7 \times 10^{-4}$	42	14
$1{\cdot}0 \times 10^{-3}$	55	—
$1{\cdot}2 \times 10^{-3}$	—	46
$1{\cdot}7 \times 10^{-3}$	90	66

Table 4. *Slopes of calibration curves*

The slopes with bacterial extract and liver extract are derived from the data of Fig. 3. For the others data were used from similar experiments without inhibitor or with 0·25 ml. of $Na_2S,9H_2O$ or 0·5 ml. of deproteinized brain extract to give the concentrations indicated.

Inhibitor	Concn. of added $CuSO_4$ (M)	Slope of calibration curve (scale units/μg. of P)	Range of added P (μg.)
None	0	2·68	0–24
	$1{\cdot}7 \times 10^{-4}$	3·95	0–20
Sodium sulphide ($1{\cdot}3 \times 10^{-6}$ M)	0	0·03	0–24
	$8{\cdot}4 \times 10^{-7}$	2·46	0–12
	$4{\cdot}2 \times 10^{-6}$	2·65	0–24
Deproteinized bacterial extract (0·83%; containing 5 μg. of P)	$4{\cdot}2 \times 10^{-6}$	2·72	0–24
Deproteinized brain extract [1·7% (v/v); containing 5 μg. of P]	$4{\cdot}2 \times 10^{-6}$	2·72	0–16
Deproteinized liver extract [1·7% (v/v); containing 12 μg. of P]	$4{\cdot}2 \times 10^{-6}$	2·34	0–16
	$8{\cdot}4 \times 10^{-6}$	2·72	0–16

small with $CuSO_4$ concentrations of $3{\cdot}3 \times 10^{-4}$ M and below (Table 3; curves *a*, *b*, Fig. 2), but with $6{\cdot}7 \times 10^{-4}$ M and above it became very large, accounting for a large proportion of the colour intensity developing in the presence of phosphate. In addition, the time course of colour development became more complex at these higher concentrations (curves *C*, *c*, Fig. 2), an initial rapid development of colour being followed by a sharp drop in intensity and then a prolonged rise, both in the presence and the absence of phosphate. The actual readings obtained were not readily reproducible (cf. the 10 min. readings in the presence of $1{\cdot}7 \times 10^{-3}$ M-$CuSO_4$ and 4 μg. of P in Fig. 2 and Table 3). The colour developing in the absence of phosphate was blue, but of a somewhat more greenish hue than in the presence of phosphate and a low concentration of copper ions; the colour of $CuSO_4$ itself at these concentrations was negligible. No adequate explanation for the above effects can be offered.

Effect of other metal ions on phosphomolybdate reduction. Colour development was unaffected by $MgSO_4$, $CoSO_4$, $NiSO_4$, $FeSO_4$ and $FeCl_3$ at $1{\cdot}7 \times 10^{-3}$ M, apart from slight inhibitions in some cases. At $1{\cdot}7 \times 10^{-6}$ M and $1{\cdot}7 \times 10^{-5}$ M, $SnCl_2$ had no effect upon the rate of phosphomolybdate reduction, though at higher concentrations the phosphomolybdate was reduced by the $SnCl_2$ itself. These experiments confirmed that the effects observed with $CuSO_4$ were indeed due to cupric rather than sulphate ions.

Reduction of phosphomolybdate by cuprous ions. It seems probable that copper ions catalyse the reduction of phosphomolybdate according to reactions 1 and 2:

$$\text{Phosphomolybdate} + Cu^{+} \rightarrow \text{reduced phosphomolybdate} + Cu^{2+}, \quad (1)$$

$$\text{Reduced ascorbic acid} + Cu^{2+} \rightarrow \text{dehydroascorbic acid} + Cu^{+}. \quad (2)$$

Reaction 1 has been known for a considerable time and has in fact been used by Folin & Wu (1920) in the determination of reducing sugars. We have established that it occurs under the conditions used for phosphate assay by treating phosphate plus molybdate with cuprous chloride reagent (see Methods). A marked blue colour developed almost instantaneously. The chromogen could be extracted

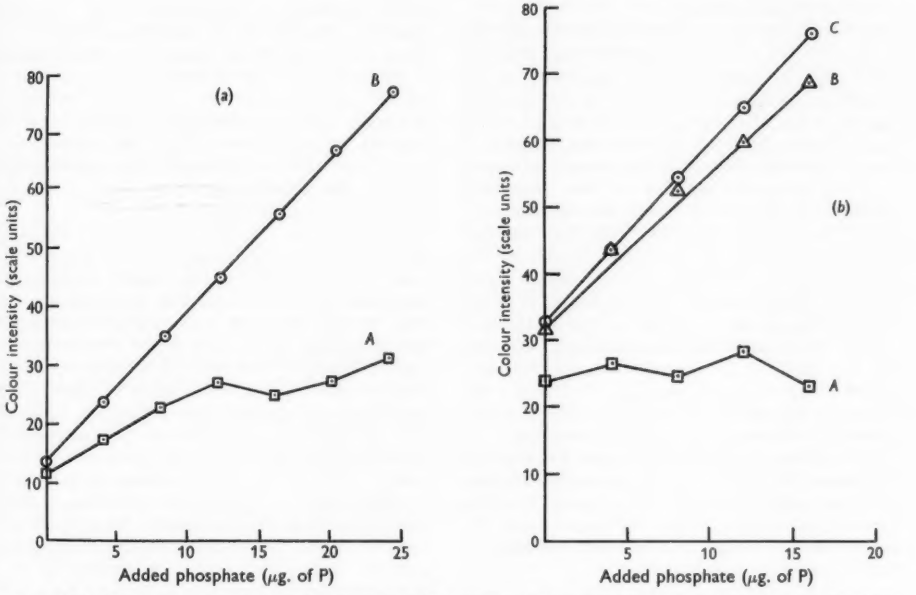

Fig. 3. Effect of copper ions on colour development in the presence of bacterial and liver extracts. Tubes contained: KH_2PO_4 (40 μg. of P/ml.), 0·5 ml. of $CuSO_4$ and 0·5 ml. of soln. of deproteinized bacterial extract or deproteinized liver extract to give the amounts indicated; ascorbic acid, ammonium molybdate and acetate buffer were present as in Fig. 1. Readings were taken after 10 min. (*a*) 0·83% of deproteinized bacterial extract present. Curve *A*, no $CuSO_4$; curve *B*, $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$. (*b*) 1·7% (v/v) of deproteinized liver extract present. Curve *A*, no $CuSO_4$; curve *B*, $4{\cdot}2 \times 10^{-6}$ M-$CuSO_4$; curve *C*, $8{\cdot}4 \times 10^{-6}$ M-$CuSO_4$.

with *iso*butanol after acidifying to pH 1 with HCl (cf. Berenblum & Chain, 1938) and was not formed when phosphate was absent, confirming that it was reduced phosphomolybdate. A slow reduction of phosphomolybdate was also observed when a few milligrams of cuprous oxide powder were used as the reducing agent.

Effect of added cupric ions in the presence of inhibitors of phosphomolybdate reduction. If copper ions are essential for the reduction of phosphomolybdate by ascorbic acid it would be expected that the inhibition by sulphide, presumed to be due to metal-binding, might be overcome by adding sufficient copper ions. This proved to be the case. With $1{\cdot}3 \times 10^{-6}$M-Na_2S, the inhibition could be overcome by adding $4{\cdot}2 \times 10^{-6}$M-$CuSO_4$, in the presence of which the calibration curve was linear over the range 0–24 μg. of P and the slope was restored to the value observed in the absence of inhibitor and added $CuSO_4$ (Table 4). With one-fifth of the same concentration of copper ions the curve was only linear over the range 0–12 μg. of P.

We have not studied the effect of copper ions on the inhibitions due to other metal-binding agents, but Bruemmer & O'Dell (1956) have shown that $6{\cdot}9 \times 10^{-5}$M-$CuSO_4$ counteracts the inhibitory effect of $8{\cdot}3 \times 10^{-5}$M glutathione.

The possibility of reversing the inhibitions observed with the biological extracts was next explored. The experiments were complicated by the presence of inorganic phosphate in the extracts themselves, but the slopes of the calibration curves (Fig. 3, Table 4) provide a convenient criterion of effective reversal. The inhibition caused by deproteinized bacterial extract (Fig. 3*a*) was completely overcome by $4{\cdot}2 \times 10^{-6}$M-$CuSO_4$ and qualitatively similar results were obtained with deproteinized brain extract. With the latter the inhibition was relatively slight, the maximum inhibition being 39 % with 16 μg. of added P. This same concentration of $CuSO_4$ was not quite adequate with the liver extract, but $8{\cdot}4 \times 10^{-6}$M was completely effective (Fig. 3*b* and Table 4). Bruemmer & O'Dell (1956), using $6{\cdot}9 \times 10^{-5}$M $CuSO_4$ and 5 μg. of P in 7·2 ml., observed a reversal of the inhibition due to a concentration of liver extract similar to our own.

The inhibition with bacterial extract was less than that observed with the amount of sulphide known to be originally present in the extract. This discrepancy may be due to traces of copper ions in the deproteinized extract, or to losses of sulphide by oxidation during its preparation.

DISCUSSION

The principal finding reported here is that copper ions counteract the inhibitory effect of sulphide and biological extracts upon the reduction of phosphomolybdate by ascorbic acid under the conditions of Lowry & Lopez (1946). A similar reversal of inhibitions due to glutathione and liver extract has been reported independently by Bruemmer & O'Dell (1956). Both investigations demonstrate the desirability of adding copper ions to the assay system for phosphate devised by Lowry & Lopez.

The strong inhibitions which we have observed with metal-binding agents indicate that a metal is required for phosphomolybdate reduction, probably as an intermediate electron carrier, and suggest that the inhibitions with sulphydryl agents, including cysteine and glutathione, are due to binding of the metal. The inhibitions with tissue extracts may be attributed to the same cause, since not only cysteine and glutathione, but also many other tissue constituents, are known to form complexes with metals (see Albert, 1950, 1952, 1953). Our results suggest that the metal concerned is copper and that the reduction observed in the absence of added copper ions is due to traces present as impurity in the reagents. The evidence, however, is entirely circumstantial: attempts to demonstrate an absolute requirement for copper ions were abandoned owing to difficulties encountered in trying to free the reagents of copper.

The reversal by copper ions of the inhibitions caused by cysteine and glutathione is interpreted by Bruemmer & O'Dell as being due to a stimulation of the oxidation of these sulphydryl compounds in air, to give the inactive disulphide derivatives. Although such an effect may contribute to the reversal, it would seem likely from the present study that the saturation of the metal-binding agent by the added copper ions is also an important, if not the major, factor involved.

Our results show that in applying the method, the concentration of free copper ions must not be too high; above $3{\cdot}3 \times 10^{-4}$M the reagent blank is very large and the course of colour development complex. Somewhat lower concentrations may have a useful application because of the increased sensitivity (50 % at $1{\cdot}7 \times 10^{-4}$M). Under these conditions, however, the reagent blank must be corrected for and a rigid timing schedule is obligatory. In general it is preferable to avoid these disadvantages by keeping the concentration of free copper ions below about 2×10^{-5}M. A concentration of $4{\cdot}2 \times 10^{-6}$M was sufficient to counteract the inhibitions observed with bacterial and brain extracts, though not quite sufficient with liver extract. Bearing in mind that in most applications such high concentrations of tissue extracts are not likely to be met, $4{\cdot}2 \times 10^{-6}$M cupric sulphate is suggested as adequate for most purposes. This concentration was found to have no effect upon the hydrolysis of acetyl phosphate under the assay conditions, the liberation of inorganic phosphate being used as a measure of the

rate of hydrolysis, and Bruemmer & O'Dell (1956) found that $6{\cdot}9 \times 10^{-5}$M cupric sulphate had no effect upon the hydrolysis of creatine phosphate.

With a little experience it is possible to judge visually whether sufficient copper ions are present, since if this is so there is a rapid development of colour within the first minute in the presence of inorganic phosphate. If not, more copper must be used, but with high concentrations of metal-binding substances it may prove difficult to ensure that the optimum concentration range of free copper ions is not exceeded. Where doubt exists, the setting up of an internal standard as suggested by Lowry & Lopez (1946) is the only true criterion as to whether the method is working satisfactorily. These points seem worth emphasizing in view of the widespread use of metal-binding agents in enzyme studies and of inorganic phosphate measurements as an index of enzymic activity.

The fact that copper is also necessary for the oxidation of ascorbic acid suggests that the requirement for copper ions in phosphomolybdate reduction may be peculiar to the use of ascorbic acid as the reducing agent. As far as we are aware, there has been no demonstration of a comparable metal requirement in the reduction of phosphomolybdate by other organic reducing agents, and we have not carried out any experiments specifically directed towards testing this point. We have, however, observed that $1{\cdot}3 \times 10^{-6}$M sodium sulphide, which completely inhibits phosphomolybdate reduction by ascorbic acid, has no effect upon reduction by 1-amino-2-naphtholsulphonic acid under the conditions of Fiske & Subbarow (1925). In view of the extremely low solubility products of cupric and cuprous sulphides ($8{\cdot}5 \times 10^{-45}$ and 2×10^{-47} at 18°) the concentration of free copper ions remaining in solution in the presence of $1{\cdot}3 \times 10^{-6}$M sodium sulphide is negligible, even at the lower pH used by Fiske & Subbarow. Copper ions do not therefore appear to be involved in the reduction of phosphomolybdate by 1-amino-2-naphtholsulphonic acid. In any case the absence of any effect with sulphide in the Fiske & Subbarow estimation indicates that the method is not susceptible to interference by metal-binding agents.

Table 5. *Use of the modified method to determine inorganic phosphate in the presence of bacterial extract and acetyl phosphate*

Mixtures for analysis were contained in a total volume of 1 ml.: 0·1M triethanolamine–HCl buffer, pH 6·8, 0·2 ml. of untreated extract of organism LC1, KH_2PO_4 and 32 μ-moles of lithium acetylphosphate were present as indicated. Bacterial extract, where present, was added last and the mixture immediately deproteinized; 0·25 ml. samples of supernatant were taken for assay. In each experiment the experimental values due to added KH_2PO_4 were obtained by subtracting from the corresponding total values the total value found in the absence of added KH_2PO_4.

	Inorganic phosphate (μmoles)		
		Experimental values	
	Added as KH_2PO_4	Total	Due to added KH_2PO_4
With bacterial extract	0	5·2	—
	6·5	12·0	6·8
	12·9	18·4	13·2
With acetyl phosphate	0	7·7	—
	6·5	14·1	6·4
	12·9	21·1	13·4
With bacterial extract plus acetyl phosphate	0	12·0	—
	6·5	18·7	6·7
	12·9	24·9	12·9

Modified method

The following modified procedure is suggested. The sample is first deproteinized with the ammonium sulphate–acetate reagent as described by Lowry & Lopez (1946), and to a portion of the resulting solution are added 0·5 ml. of 5×10^{-5}M $CuSO_4$ and 0·125M sodium acetate buffer, pH 4, to a total volume of 5 ml., followed by 0·5 ml. of 1% ascorbic acid. The reduction is then started by the addition of 0·5 ml. of 1% ammonium molybdate in 0·05N-H_2SO_4, and the colour intensity is measured after 10 min. When labile phosphates are present, readings are taken at 10 and 20 min., and the colour intensity corresponding to the inorganic phosphate present in the original sample is obtained by extrapolation to zero time. The taking of readings at 10 and 20 min. was found to be more suitable than at the times of 5 and 10 min. suggested by Lowry & Lopez (1946). A longer time is thereby allowed for completion of the reaction with the free phosphate.

The application of this modified procedure in the presence of bacterial extract and acetyl phosphate is illustrated in Table 5. Both the bacterial extract and the acetyl phosphate preparation contained free phosphate, but after correcting for these by subtraction, the recovery of inorganic phosphate was considered satisfactory, bearing in mind that four experimental readings were involved in the determination of each figure for recovered inorganic phosphate.

SUMMARY

1. The reduction of phosphomolybdate to a blue compound by ascorbic acid under the conditions of Lowry & Lopez (1946) is inhibited by extracts of a rumen micro-organism and by liver and brain extracts.

2. Strong inhibitions are observed in the presence of micromolar concentrations of sodium sulphide, diethyldithiocarbamate and ethylenediaminetetra-acetic acid and in the presence of millimolar concentrations of cysteine.

3. At low concentrations (e.g. 2×10^{-6}M) cupric ions cause a marked acceleration of the rate of colour development. At higher concentrations

($1{\cdot}7 \times 10^{-4}$ M) the final colour attained is also increased, and above this concentration a marked colour appears in the absence of phosphate. No comparable effects are observed with Mg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+} or Sn^{2+} ions.

4. Cuprous ions reduce phosphomolybdate to the blue complex under the conditions for phosphate assay. It is suggested that copper ions act as electron carriers in the reduction of phosphomolybdate by ascorbic acid and that normally sufficient copper is present as impurity in the reagents. In the presence of metal-binding agents, including those occurring in tissues, phosphomolybdate reduction is inhibited as a result of metal-binding action.

5. The addition of $4{\cdot}2 \times 10^{-6}$ M copper ions to the system overcomes the effect of such concentrations of metal-binding agents as are likely to be met with in normal practice. A suitable modification to the procedure of Lowry & Lopez is described.

6. By using a somewhat higher concentration ($1{\cdot}7 \times 10^{-4}$ M) of copper ions, the sensitivity of the method can be increased by 50%, though the timing of operations becomes more critical under these conditions. Concentrations above $3{\cdot}3 \times 10^{-4}$ M interfere with the method.

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Hydrolysis of Amides by Extracts from Mycobacteria

By Y. S. Halpern and N. Grossowicz
Department of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Amidases of bacterial origin have received less attention than amidases of animal origin. Amidase systems in micro-organisms which catalyse the hydrolysis of glutamine (Hughes & Williamson, 1952; McIlwain, 1948), asparagine (Altenbern & Housewright, 1954) and nicotinamide (Hughes & Williamson, 1953; Oka, 1954) have been described. Altenbern & Housewright (1954) were able to show the existence of two stereospecific asparaginases in extracts from *Brucella abortus*. Kirchheimer & Whittaker (1954) reported the presence of asparaginase activity in various species of Mycobacteriaceae.

This study deals with amidase activities of *Mycobacterium phlei* and *Mycobacterium tuberculosis* var. *bovis* B.C.G. and with the substrate specificities of the enzymes studied.

EXPERIMENTAL

Micro-organisms. The organisms used were *Mycobacterium phlei* 271 from the culture collection of this Department and *Mycobacterium tuberculosis* var. *bovis* B.C.G. 5692 (B.C.G.) obtained from the American Type Culture Collection (ATCC).

Cultivation of bacteria. The *Myco. phlei* strain was grown for 4 days at 35° in Roux bottles on nutrient agar supplemented with 0·1% Tween 80 (polyoxyethylene sorbitan mono-oleate). The bacteria were taken up in water, washed 3 times by centrifuging and then resuspended in water. The B.C.G. strain was grown in Sauton's medium (Sauton, 1912) for about a month at 35°. The pellicles were filtered off, washed three times with water and a thick suspension of washed cells was prepared.

Bacterial extracts. Cell-free extracts were prepared by disintegrating the washed bacteria in a 9 kcyc./sec. Raytheon sonic vibrator for 25 min. The cell debris was removed in the

cold by centrifuging in a Servall centrifuge at 10 000 *g* for 20 min. The cell-free extracts were then dialysed overnight against distilled water or running tap water at 6°. The protein content of the extracts was determined by Mehl's biuret method (Mehl, 1945).

Ammonia determination. Ammonia was determined either by Conway's microdiffusion method or colorimetrically by nesslerization of samples previously distilled into 0·1 N-HCl. Distillation was carried out for 40 min. in a water bath at 50° under a constant air current.

Amino acid determination. Amino acids were identified by paper chromatography on sheets of Whatman no. 2 filter paper with water-saturated phenol as the solvent. A 0·1% ninhydrin solution was used as the developing reagent. For quantitative determination of amino acids from paper chromatograms the method of Giri, Radhakrishnan & Vaidyanathan (1952) was employed.

RESULTS

Amidase activities of extracts of Myco. phlei *and B.C.G.* Preliminary screening revealed that cell-free extracts of *Myco. phlei* catalysed the hydrolysis of L-asparagine, glycinamide, DL-leucinamide, DL-valinamide, formamide and nicotinamide. Glutaminase activity, when present, was very weak compared to that of asparaginase. Acetamide was hydrolysed to a small extent, whereas benzamide and phenylalaninamide were not.

Extracts of B.C.G., like *Myco. phlei* preparations, showed considerable amidase activity towards L-asparagine, DL-leucinamide and DL-valinamide, and only weak activity towards L-glutamine; glycinamide, formamide, nicotinamide and phenylalaninamide were not attacked. Acetamide and benzamide were not tested. These results are summarized in Table 1.

Asparaginase activity of Myco. phlei *extracts.* Dialysis of the *Myco. phlei* cell-free preparation against distilled water did not appreciably affect the activity of the asparaginase system. The presence of 0·035 M-KBr (Hughes & Williamson, 1952) or of 0·02 M pyruvate (Greenstein & Carter, 1946) did not influence the rate of hydrolysis of asparagine. These treatments did not increase the glutaminase activity.

The influence of hydrogen-ion concentration on the asparaginase activity of *Myco. phlei* was studied. As can be seen from Fig. 1, the enzyme is active

Table 1. *Hydrolysis of amides by extracts from* Myco. phlei *and* Myco. tuberculosis *B.C.G.*

The system contained: bacterial extract (3 mg. of protein), substrate as indicated, phosphate buffer, pH 8·3 (0·02 M); total volume, 1 ml.; incubation at 37° for 2½ hr.

Substrates		NH_3 formed (μmoles)	
Nature	Amount (μmoles)	With *Myco. phlei* extracts	With B.C.G. extracts
L-Glutamine	10	0·3	1·1
L-Asparagine	10	2·8	9·6
Glycinamide	10	5·0	0·5
DL-Leucinamide	20	9·0	8·7
DL-Valinamide	20	10·3	9·3
DL-Phenylalaninamide	20	0	0
Nicotinamide	10	6·0	0
Benzamide	20	0	—
Formamide	20	5·2	0·1
Acetamide	20	1·0	—

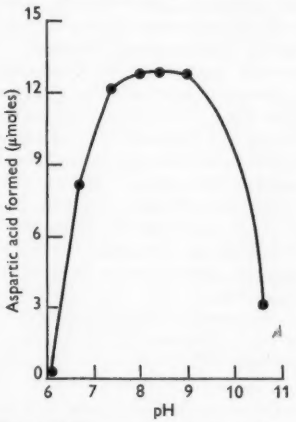

Fig. 1. Effect of pH on asparaginase activity in *Myco. phlei* extracts. The system contained: 17·6 μmoles of L-asparagine, bacterial extract (5·8 mg. of protein), phosphate buffer (0·013 M); total volume, 1 ml.; incubation at 37° for 4 hr.

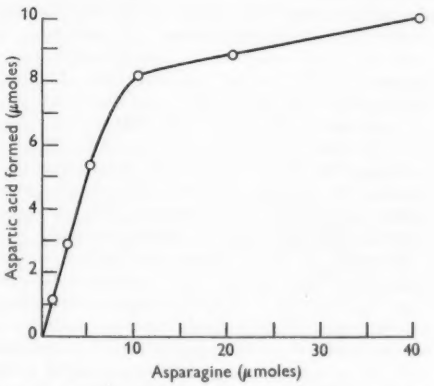

Fig. 2. Influence of substrate concentration on asparaginase activity of *Myco. phlei* extracts. The system contained: L-asparagine in varying concentrations (0–40 μmoles), bacterial extract (2·5 mg. of protein), phosphate buffer, pH 8·2 (0·007 M); total volume, 1 ml.; incubation at 37° for 1½ hr.

over a wide range of pH values (6·5–10·5) with an optimum at pH 8·0–9·0.

The rate of hydrolysis of asparagine by the *Myco. phlei* preparation was examined. At 37°, the rate of aspartic acid formation from asparagine remained constant for more than 3 hr.

Fig. 2 shows the relationship between the initial concentration of L-asparagine and the rate of formation of aspartic acid. At a concentration of 10 μmoles/ml. the reaction rate was almost maximum and increased only slightly with further addition of substrate (up to 40 μmoles/ml.).

Substrate specificity of asparaginase. A series of analogues and derivatives of L-asparagine were tested as potential substrates for the enzyme. As can be seen from Table 2 none of the eight compounds tested was deamidated by the asparaginase of *Myco. phlei*, with the exception of the dipeptide, glycyl-L-asparagine, which was hydrolysed to glycine, aspartic acid and ammonia. This finding indicated the presence in the *Myco. phlei* extracts of a peptidase which was catalysing the hydrolysis of the dipeptide. The question arose as to whether the asparaginase could deamidate the intact peptide or whether prior hydrolysis of the dipeptide to its monomers was essential. Since the activity of some peptidases was shown to be dependent on the presence of metal ions (Smith, 1949), ethylenediaminetetraacetic acid (EDTA) was used to elucidate this point. It was found that EDTA inhibited to the same extent the liberation of glycine and ammonia from the peptide but did not inhibit ammonia formation from L-asparagine (Table 3). Paper chromatography revealed spots of aspartic acid, glycine and the residual amount of glycyl-L-asparagine, when EDTA was added to the system containing glycyl-L-asparagine as substrate; no other spot was seen, hence glycyl-L-aspartic acid appeared to be absent. On the other hand, D-asparagine, a specific inhibitor of the *Myco. phlei* asparaginase (Grossowicz & Halpern, 1956*a*), inhibited the liberation of ammonia from the dipeptide, but had no effect on the formation of glycine. It appears therefore that asparaginase cannot attack the intact peptide unless the latter is first hydrolysed to glycine and asparagine.

Inhibition of asparaginase by analogues of L-asparagine. Those analogues of L-asparagine which were inactive as substrates were subsequently tested for inhibitory effects on the asparaginase system (Table 4). All the analogues of L-asparagine with an unsubstituted amide group acted as inhibitors. However, substitution of one hydrogen atom in the amide radical by a propyl or butyl group abolished the inhibitory effect of the analogue.

Differentiation between amidases of Myco. phlei. The remarkable specificity of the asparaginase studied suggested that the various amidase activities

Table 2. *Substrate specificity of asparaginase in extracts from* Myco. phlei

The reaction mixture contained: 10 μmoles of substrate, bacterial extract (3·5 mg. of protein), phosphate buffer, pH 8·2 (0·033 M); total volume, 1 ml.; incubation at 37° for 2 hr.

Substrate	NH_3 formed (μmoles)
L-Asparagine	8·50
D-Asparagine	0·12
N^2-Ethyl-DL-asparagine	0·02
Glycyl-L-asparagine	7·60
Glycyl-D-asparagine	0
DL-Alanyl-DL-asparagine	0·50
DL-α-Asparagine (*iso*asparagine)	0·40
Maleamic acid	0·22
N-*n*-Butyl-DL-asparagine	0·20*

* Splitting of *N*-*n*-butyl-DL-asparagine by asparaginase should yield aspartic acid. No evidence (by paper chromatography) for aspartic acid formation was found.

Table 3. *Influence of ethylenediaminetetraacetic acid (EDTA) and D-asparagine on the hydrolysis of L-asparagine and glycyl-L-asparagine by extracts from* Myco. phlei

The system contained: bacterial extract (3·6 mg. of protein), 10 μmoles of substrate as indicated, 0 or 50 μmoles of EDTA, 0 or 40 μmoles of D-asparagine, phosphate buffer, pH 8·2 (0·007 M); total volume, 1 ml.; incubation at 37° for 2½ hr. All values are given after correction for controls (substrate without extract, extract without additions, extract with D-asparagine alone).

	Formation of NH_3 and glycine in the presence of inhibitors, expressed as % of control system without inhibitor			
	EDTA present		D-Asparagine present	
Substrate	NH_3	Glycine	NH_3	Glycine
Glycyl-L-asparagine	57·9	61·0	17·5	100·0
L-Asparagine	98·0	—	19·0	—

Table 4. *Inhibition of asparaginase by analogues of L-asparagine*

The reaction mixture contained: 10 μmoles of L-asparagine, bacterial extract (2·3 mg. of protein), inhibitor as indicated (40 μmoles), phosphate buffer, pH 8·2 (0·007 M), total volume, 1 ml.; incubation at 37° for 3 hr. All values are given after correction for controls (enzyme without substrate, substrate alone and enzyme with inhibitor only).

Inhibitor added	Inhibition (%)
D-Asparagine	81
N^2-Ethyl-DL-asparagine	34
Glycyl-D-asparagine	84
DL-Alanyl-DL-asparagine	66
DL-α-Asparagine (*iso*asparagine)	25
N-*n*-Butyl-DL-α-asparagine	0
Maleamic acid	31
N-*n*-Propyl-maleamic acid	0
N-*n*-Butyl-DL-asparagine	0

Table 5. *Effect of heating in the presence of substrates on the amidase activities of* Myco. phlei *extracts*

The system contained: 30 μmoles of L-asparagine or nicotinamide or 10 μmoles of glycinamide, bacterial extract (5·3 mg. of protein) after heating at 65° for 15 min. in the presence of 50 μmoles of the appropriate substrate and subsequent dialysis to remove the substrate, phosphate buffer, pH 8·2 (0·017 M); total volume, 1 ml.; incubation at 37°: asparaginase system for 1 hr., nicotinamidase for 30 min. and glycinamidase for 10 min.

Treatment of extract	NH_3 formed (μmoles) from L-Asparagine	Glycinamide	Nicotinamide
Untreated (control)	4·4	4·1	5·5
Heated in the absence of substrate	0	3·3	3·3
Heated in the presence of L-asparagine	1·9	3·2	2·1
Heated in the presence of glycinamide	0·3	3·0	2·3
Heated in the presence of nicotinamide	0·3	3·0	4·6

of *Myco. phlei* might be due to different enzymes. Evidence for this hypothesis was provided by the selective inactivation of the asparaginase upon storage. The extracts were normally used within 2–3 days after preparation. Storage for 2–4 weeks at 0° caused a considerable decrease (50 % or more) in asparaginase activity without affecting appreciably the amidases of nicotinamide, glycinamide and leucinamide. Further evidence was provided by three different approaches: (*a*) selective protection of enzymes against heat inactivation by the addition of the corresponding substrates; (*b*) selective inhibition of enzyme activity by substrate analogues; (*c*) the effect of addition of a second amide (as measured by ammonia production) to an enzyme system already saturated with one amide.

(*a*) Three samples of the enzyme preparation were incubated at 65° for 15 min. in the presence of asparagine, glycinamide and nicotinamide respectively. After dialysis for 18 hr. the activity of each sample towards the three substrates was determined. For comparison, the activities of an untreated extract and of extract heated in the absence of substrate were determined simultaneously. Results of a typical experiment are shown in Table 5. It is quite evident that the enzymes are selectively protected from heat inactivation by the presence of the corresponding amides. Thus, for example, the presence of asparagine during the heating of the extract protected the asparaginase but not the nicotinamidase. It can also be seen that in the absence of the substrate, the asparaginase is much more susceptible to heat (65° for 15 min.) than are the nicotinamidase and glycinamidase activities. In other experiments, under slightly different conditions (temperature and time of exposure, concentration of protecting substrate, etc.), essentially similar results were obtained. For example, asparaginase activity was completely destroyed when heated at a slightly lower temperature (59° for 15 min.) in the absence of asparagine, regardless of the presence of glycinamide or nicotinamide. The presence of asparagine during heating resulted in the preservation of 90 % of the original activity. On the other hand, under the same conditions the glycinamidase activity was only slightly affected (20 %); this small inactivation was not prevented by the presence of glycinamide or of the other substrates.

Table 6. *Effect of* D-*asparagine on the hydrolysis of other amides by extracts from* Myco. phlei

The system contained: substrate as indicated, bacterial extract (3·8 mg. of protein), phosphate buffer, pH 8·2 (0·007 M); total volume, 1 ml.; incubation at 37° for 2½ hr. All values are given after correction for controls (enzyme without substrate, enzyme with D-asparagine, substrate without enzyme).

Substrate Nature	Amount (μmoles)	NH_3 formed (μmoles) No inhibitor added	30 μmoles of D-asparagine added
L-Asparagine	10	8·9	4·0
Glycinamide	10	4·0	3·8
Formamide	10	8·4	8·5
DL-Leucinamide	20	8·9	9·1
DL-Valinamide	20	8·3	7·9
Nicotinamide	10	9·2	9·4

(*b*) The second method was based on the assumption that if asparagine, nicotinamide and other amides were hydrolysed by the same enzyme, a substance inhibitory to one of these reactions (e.g. splitting of asparagine) would inhibit the hydrolysis of the other substrates (e.g. nicotinamide). Previous findings have shown (Grossowicz & Halpern, 1956*a*) that D-asparagine inhibits competitively the hydrolysis of L-asparagine in extracts of *Myco. phlei*. The effect of D-asparagine on the hydrolysis of other amides was therefore tested. The data presented in Table 6 show that D-asparagine had no effect on the deamidation of substrates other than L-asparagine. Similarly, 3-acetylpyridine inhibited competitively the deamidation of nicotinamide, but had no effect upon the hydrolysis of other amides tested (Grossowicz & Halpern, 1956*b*).

(*c*) Further addition of glycinamide to an enzyme system already saturated with this substrate did not increase the rate of ammonia production. On

Table 7. *Effect of addition of one amide to an enzyme system saturated with another amide*

The system contained: varying amounts of one substrate (20–60 μmoles), or a mixture of nicotinamide and glycinamide (30–60 μmoles of each), *Myco. phlei* extract (2·6 mg. of protein), phosphate buffer, pH 8·2 (0·007 M); total volume, 1 ml.; incubation at 37° for 2½ hr.

Substrate added		
Nature	Amount (μmoles)	NH_3 formed (μmoles)
Nicotinamide	20	4·2
Nicotinamide	30	4·7
Nicotinamide	40	4·8
Nicotinamide	60	5·4
Glycinamide	20	2·7
Glycinamide	30	3·4
Glycinamide	40	4·0
Glycinamide	60	4·4
Nicotinamide +glycinamide	30 30	8·8
Nicotinamide +glycinamide	60 60	10·1

the other hand, addition of a different substrate (e.g. nicotinamide) to the same system doubled the amount of ammonia formed (Table 7).

DISCUSSION

The specificity of the asparagine deamidase of *Myco. phlei* is very remarkable. The experiments reported here seem to indicate that the free α-amino and amide groups, as well as the proper optical configuration of the substrate, are essential for asparaginase activity. Substitution of one hydrogen in the α-amino group of asparagine abolishes the activity of the compound as a substrate and transforms it into an inhibitor, provided that the amide amino group remains unsubstituted. Maleamic acid, which has no α-nitrogen, also inhibits the hydrolysis of L-asparagine, but *N*-*n*-propylmaleamic acid (with a substituted amide group) is without any effect. D-Asparagine (the unnatural enantiomorph of L-asparagine) and *iso*asparagine (with the amino group adjacent to the amide group) proved to be specific inhibitors of the enzyme. L-Asparagine and *iso*asparagine substituted in the amide-amino group are inactive, both as substrates and as inhibitors.

The selective inhibition of asparaginase and nicotinamidase by substrate analogues, the selective protection against heat inactivation in the presence of the corresponding substrate and the 'addition effect' caused by the simultaneous presence of two different substrates, indicate quite clearly the presence of several distinct amidases. On the basis of the data presented it is suggested that *Myco. phlei* possesses at least three different amidases: L-asparaginase, nicotinamidase and a third enzyme which catalyses the hydrolysis of glycinamide. Whether the hydrolysis of glycinamide, leucinamide, valinamide and formamide is catalysed by the same or different enzymes has yet to be investigated.

SUMMARY

1. The amidase activity of extracts from *Mycobacterium phlei* and *Mycobacterium tuberculosis* (B.C.G.) were studied. Extracts from either of the two species catalysed the hydrolysis of L-asparagine, leucinamide and valinamide; a slight activity was found towards L-glutamine and no activity at all towards phenylalaninamide. Extracts from *Myco. phlei* but not from B.C.G. were also active towards glycinamide, formamide and nicotinamide, but did not hydrolyse benzamide.

2. The kinetics and substrate specificity of the *Myco. phlei* asparaginase were investigated. The enzyme attacked L-asparagine only and was inactive towards D-asparagine, *iso*asparagine, maleamic acid or L-asparagine substituted in the α-amino or amide-amino groups.

3. D-Asparagine and other analogues of L-asparagine (*iso*asparagine, maleamic acid and N^2-ethyl-DL-asparagine) inhibited the asparaginase activity. A free amide-amino group is essential for rendering the substance inhibitory.

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A Modified Glutamic Acid Dehydrogenase as a Result of Gene Mutation in *Neurospora crassa*

By J. R. S. FINCHAM
Department of Genetics, University College, Leicester

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Previous papers (Fincham, 1950, 1954) have been concerned with mutant strains of *Neurospora crassa* which appear completely to lack glutamic acid dehydrogenase and which require one of a number of alternative L-α-amino acids for normal growth. More recently, Pateman (1957) has studied ultra-violet-light-induced reverse mutations in these strains. Most of the apparent back-mutants proved to have regained the capacity to synthesize glutamic dehydrogenase in normal amounts, but several strains, although growing more or less like the wild type, gave exceptionally low glutamic dehydrogenase activities in crude extracts. One of these latter strains is the subject of this paper. It produces an abnormal glutamic dehydrogenase distinguished from the normal *Neurospora* enzyme by its capacity for reversible activation; activation can be brought about by mild heat treatment or, to some extent, by incubation with substrates.

The mutation resulting in the production of the abnormal enzyme has been shown to be at the same locus as the original mutation (associated with apparent absence of glutamic dehydrogenase) from which it was secondarily derived (Fincham & Pateman, 1957).

MATERIALS AND METHODS

Neurospora *strains*. In this paper the term 'mutant' will be used to refer to strains carrying the gene for abnormal glutamic dehydrogenase. Several different mutant and wild-type strains were isolated in the course of the analysis of a series of crosses stemming from the standard wild-type strain St Lawrence *A* (St.L.*A*) and the original mutant, isolated by Pateman, no. 689. Strains used for enzyme preparations are listed in Table 1. The detailed derivation of the strains is given elsewhere (Fincham & Pateman, 1957).

Enzyme preparations. Cultures were grown from heavy conidial inocula for 48 hr. at 25° without agitation in 150 ml. lots of N-free buffered medium (Fincham, 1954) supplemented with 0·01 M ammonium tartrate. The mycelium was collected by filtration, thoroughly washed with glass-distilled water, blotted to remove excess of moisture, roughly weighed, and ground in an ice-chilled mortar with powdered glass and three times its wet weight of 0·05 M phosphate (Na_2HPO_4–KH_2PO_4) buffer, pH 8·0. After grinding, the volume of buffer was increased to 12 times the original wet weight of mycelium and the blend was filtered by suction through a kieselguhr pad (Fincham, 1954). The almost clear opalescent filtrate was either dialysed with stirring against three changes of the same buffer as used in the extraction (to give 'crude extract', Table 1), or was further fractionated as follows. The filtrate was placed in an ice bath and solid $(NH_4)_2SO_4$ was added with stirring over 15 min. to give 50% saturation. The precipitate was collected by centrifuging, taken up in a volume of 0·05 M phosphate, pH 8·0, equal to about ten times the original wet weight of mycelium, and dialysed against three changes of the same buffer at 0–5°, the last change being left in the refrigerator overnight. After dialysis the preparation was centrifuged and any precipitate was discarded. Preparations were usually stored frozen at −15°; under these conditions wild-type preparations were generally fairly stable for many weeks, but most of the mutant preparations lost activity at the rate of about 50% per week. After freezing and thawing, some further precipitate often appeared which could be removed without detriment to the activity of the preparation. Dialysed fractionated preparations contained only traces of ammonia (of the order of 10^{-4} M), and were usually purified about twofold (with respect to protein) as compared with the crude extracts (Table 1). Unlike crude dialysed extracts, they did not reduce triphosphopyridine nucleotide (TPN) appreciably in the absence of glutamate. Some further purification could be achieved by refractionating between 40 and 50% saturation with $(NH_4)_2SO_4$, but the resulting preparation lost activity rapidly. A 50–80% saturation fraction had very little activity.

The protein content of enzyme preparations was determined by the biuret procedure previously described (Fincham, 1954).

Measurement of enzyme activity. A Unicam SP. 500 spectrophotometer was used to measure oxidation of reduced triphosphopyridine nucleotide (TPNH) or reduction of TPN at 340 mμ. The usual assay system consisted of a suitable dilution of enzyme in 0·08 M phosphate (Na_2HPO_4–KH_2PO_4) buffer, pH 8·0, 6·0 or $6·7 \times 10^{-3}$ M sodium α-oxoglutarate, $3·3 \times 10^{-3}$ N ammonium salt (usually $(NH_4)_2SO_4$, though NH_4Cl gave the same activity) and $5·0–6·5 \times 10^{-5}$ M TPNH, giving an E_{340} of 0·32–0·40. These components were mixed in a 1 cm. silica spectrophotometer cell, the final volume being 3 ml. The reaction was started by the addition of either TPNH or ammonium salt, followed by immediate mixing by inversion of the cell. Density readings were taken 30 sec. after the start of the reaction, and at 15 sec. intervals thereafter. With the concentrations of TPNH usually employed the reaction catalysed by the wild-type enzyme was approximately constant in rate during the first change in density of 0·1, so that rates of up to about 0·1/min. could be measured with an error of only a few per cent; initial rate was proportional to enzyme concentration. Mutant-enzyme preparations gave accelerating reaction rates under certain conditions (see the Results section). Assays were performed at room temperature, which was nearly constant

Table 1. *Summary of enzyme preparations*

In all assays the reaction was started by the addition of TPNH; activities of mutant preparations were calculated from maximum rather than initial rates.

No.	Strain	Type of preparation	Concn. in assay system (mM) α-Oxo-glutarate	Concn. in assay system (mM) NH_4^+	Heat treatment	Specific activity Untreated	Specific activity Heated
1	St.L.*A* (wild type)	Fractionated	6·0	2·5	—	1350	—
2	1361–12 (mutant)	Fractionated	6·0	2·5	—	100	—
3	1432–12 (wild type)	Fractionated	8·0	2·5	8 min., 50°	1700	1700
4	1432–34 (mutant)	Fractionated	8·0	2·5	8 min., 50°	76	370
5	St.L.*A*	Crude extract	6·7	3·3	10 min., 50°	1000	1000
	St.L.*A*	Fractionated	6·7	3·3	10 min., 50°	2100	2100
6	1432–78 (mutant)	Crude extract	6·7	3·3	10 min., 50°	60	230*
	1432–78 (mutant)	Fractionated	6·7	3·3	10 min., 50°	110	225*
7	1497–34 (mutant)	Crude extract	6·0	3·3	6 min., 50°	20	170
	1497–34 (mutant)	Fractionated	6·0	3·3	6 min., 50°	38	250
8	S5*a* (wild type)	Fractionated	6·7	3·3	5 min., 50°	1200	1200
9	1435–12 (mutant)	Fractionated	6·7	3·3	5 min., 50°	40	250
10	1435–34 (mutant)	Fractionated	6·7	3·3	6½ min., 38·5°	170	1200

* These values are probably low because of over-prolonged heat treatment.

during any one experiment and which varied between 18° and 21° from one experiment to another. All components of the system except the TPNH were kept in a water bath at about 1° above room temperature before mixing; the TPNH solution, 0·2 ml. of which was added in a final volume of 3 ml., was kept in an ice bath. Enzyme activities are expressed as $10^3 \Delta E_{340}$/min. Specific activities are expressed as activity/mg. of protein.

Chemicals. α-Oxoglutaric acid was used as supplied by L. Light and Co. Ltd., Colnbrook, Bucks. TPN (different batches of 81–98% purity, sodium salt) was the product of the Sigma Chemical Co. (U.S.A.) and was obtained through George T. Gurr Ltd. (136, New Kings Road, London, S.W. 6). TPNH was prepared in solution by reducing TPN with a dialysed extract of an acetone-dried powder of pig heart and a slight excess of DL-*iso*citrate (based on Evans & Nason, 1953) and was stored frozen at −15°.

RESULTS

Properties of the wild-type enzyme

The activity of the wild-type enzyme in 0·08 M orthophosphate (Na_2HPO_4–KH_2PO_4) buffer increased with increasing pH up to 8·0. The pH optimum, determined in 0·032 M aminotrishydroxymethylmethane (tris)–acetic acid buffer, was close to 8·5 (Fig. 1). In sodium pyrophosphate–HCl buffer the pH optimum, though less accurately determined, was again about 8·5. The activities of the enzyme in the tris–acetic acid and pyrophosphate–HCl buffers at pH 8·0 were respectively about 60 and 70% of the activity in orthophosphate at the same pH value, and the highest activity obtained was with orthophosphate at pH 8·0; accordingly, this buffer was used in all subsequent experiments both with wild-type and with mutant enzyme (the pH optimum of the mutant enzyme was similar to that of the wild-type enzyme,

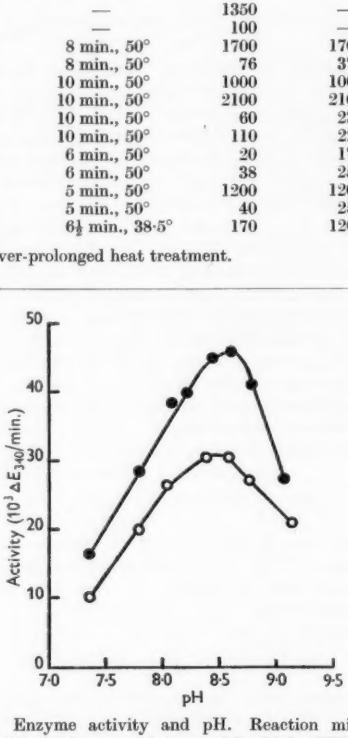

Fig. 1. Enzyme activity and pH. Reaction mixtures with $6·7 \times 10^{-3}$ M α-oxoglutarate, $3·3 \times 10^{-3}$ N-$(NH_4)_2SO_4$, 0·032 M tris–acetic acid buffer, 57 μg. of protein from wild type (preparation 8, Table 1) (●) or 400 μg. of protein from the mutant (preparation 9) (○) in 3 ml., and approx. $5·5 \times 10^{-5}$ M TPNH. Reactions were started by adding the ammonium salt in 0·2 ml. after 4 min. incubation of the enzyme with the TPNH and α-oxoglutarate; pH values were determined on reaction mixtures with a glass electrode.

Fig. 1). Experiments in which the enzyme was used to reduce TPN with glutamate showed, for this reaction also, a pH optimum of about 8·5 in pyrophosphate, but a higher activity at pH 8·0 in orthophosphate.

The enzyme gave rate–substrate concentration curves of the Michaelis–Menten type for glutamate, α-oxoglutarate and NH_4^+ ion. Michaelis constants,

determined by the methods of Lineweaver & Burk (1934), were as follows: 7·2 mM for glutamate, 3·7 mM for α-oxoglutarate, mM for NH_4^+ ion (Figs. 2–4).

Basis for the distinction between wild-type and mutant enzymes

Wild type and mutant strains were always clearly distinguished by the relatively very low glutamic dehydrogenase activities shown by crude extracts of the latter (Table 1, and Fincham & Pateman, 1957). A second distinction of a qualitative character was revealed by experiments originally designed to test the heat-stability of the two kinds of preparation. It was found that crude extracts or fractionated preparations of mutant strains increased strikingly in activity on being heated to 35–50° for 4–10 min. Except where the heating probably exceeded the optimum, increases in activity of six- to eight-fold were generally obtained (Table 1), although no absolute significance can be attached to these figures since the activity shown by untreated mutant preparations depends on the technique of measurement (see below). Wild-type preparations showed no measurable change in activity when subjected to similar heat treatment. In general, the activity of heat-treated mutant preparations still fell short of typical wild-type activity (Table 1).

Another less immediately obvious difference between wild-type and mutant-enzyme preparations was the presence of a lag phase in the reaction catalysed by mutant enzyme when the reaction was started by adding TPNH. No such lag has been observed in the wild-type enzyme under the conditions used in this series of experiments, although a lag effect, which has not been further investigated, has been noticed when wild-type enzyme has been tested at pH 7·4. The cause of the lag effect with mutant enzyme is considered below.

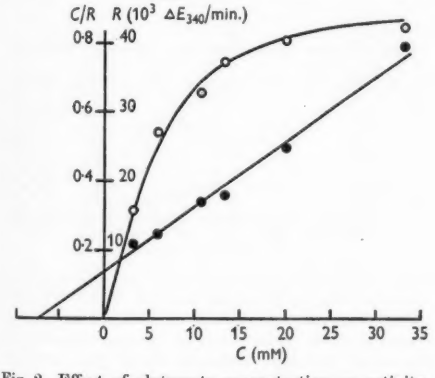

Fig. 2. Effect of glutamate concentration on activity of wild-type enzyme. Reaction mixtures with 0·06 M orthophosphate, pH 8·0, approx. 10^{-4} M TPN, and 0·25 mg. of protein of preparation 1 (Table 1). ○, Rate (R) and glutamate concentration (C); ●, C plotted against C/R for determination of the Michaelis constant (K_m, 7·2 mM).

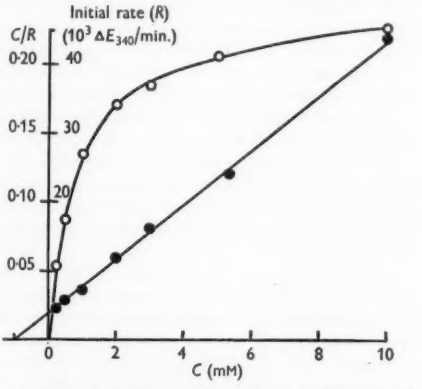

Fig. 3. Effect of NH_4^+ ion concentration on activity of wild-type enzyme. Reaction mixtures with 0·08 M orthophosphate, pH 8·0, $6·7 \times 10^{-3}$ M α-oxoglutarate, approx. $3·5 \times 10^{-5}$ M TPNH and 57 μg. of protein of preparation 8 (Table 1) in 3 ml. ○, Rate (R) and NH_4^+ ion concentration (C); ●, C plotted against C/R for determination of the Michaelis constant (K_m, mM).

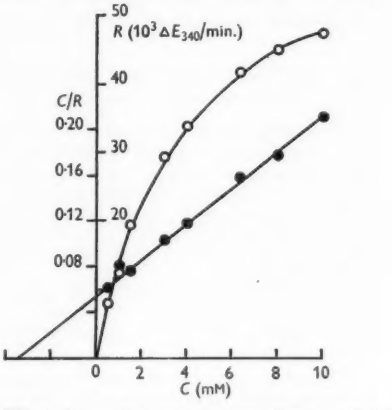

Fig. 4. Effect of α-oxoglutarate concentration on activity of wild-type enzyme. Reaction mixtures with 0·09 M orthophosphate, pH 8·0, $3·3 \times 10^{-3}$ N-$(NH_4)_2SO_4$, approx. $4·5 \times 10^{-5}$ M TPNH, and 29 μg. of protein of preparation 5 (Table 1) in 3 ml. ○, Concentration (C) and rate (R); ●, C plotted against C/R for determination of the Michaelis constant (K_m, 3·7 mM).

In general, mutant-enzyme preparations were less stable at −15° than wild-type preparations (see Methods section). It was also found that whereas wild-type preparations were quite stable at 50° for periods of up to 20 min. (preparations 3, 5 and 8, Table 1, were tested), most of the mutant preparations tested (preparations 4, 6 and 7, Table 1) showed a pronounced falling off in activity at 50° after the initial activation effect. Preparation 7, for example, lost about half its activity between 2 and 12 min. at 50°. On the other hand, preparation 9 was stable during 20 min. at 50°. Thus although there is a general indication that the mutant enzyme is less stable than the wild-type enzyme, there is much variation in this respect between different preparations. Ageing of mutant preparations did not result in any apparent qualitative change in the properties of the enzyme; in particular, the heat-activation effect was similar in fresh and old preparations.

Since the enzyme fractions used in these experiments were relatively crude it was thought possible that a difference in respect of an impurity, rather than a difference in the enzymes themselves, might account for the difference in properties of wild-type and mutant preparations. This possibility was rendered less plausible by a number of experiments on mixed mutant and wild-type preparations. Table 2 shows the results of a representative experiment. There was no inhibition of wild-type enzyme activity by the mutant preparation, nor stimulation of mutant-enzyme activity by the wild-type preparation. The effect of heat on the mixture was such as would be expected if the mutant enzyme alone was responding to the treatment.

Table 2. *Absence of interaction between wild-type and mutant-enzyme preparations*

Reaction mixtures (3 ml.) contained: 1 ml. of enzyme solution in 0·1 M phosphate, pH 8·0, containing 0·025 mg. of wild-type protein or 0·11 mg. of mutant protein or both, and final concentrations of 0·09 M phosphate, pH 8·0, $6{\cdot}7 \times 10^{-3}$ M α-oxoglutarate, $3{\cdot}3 \times 10^{-3}$ N-$(NH_4)_2SO_4$, and approx. 6×10^{-5} M TPNH. Reactions were started by adding the TPNH. Fractionated enzyme preparations 5 and 6 were used.

Nature of enzyme	$10^3 \times$ Activity (ΔE_{340}/min.) Enzyme solution not heated	Enzyme soln. heated to 50° for 10 min. immediately before testing
Wild type	51	51
Mutant	8·6*	55†
Mixture	60	110

* Showing a slightly accelerating rate; initial rate, as measured over the first 90 sec., is shown.

† Enzyme tested at half strength, and the resulting activity doubled.

Nature of the temperature activation of mutant enzyme

A complication which arose in assessing the effect of temperature treatment was due to the fact that mutant-enzyme preparations which were kept at or below 21° showed an initial lag in reaction rate when the reaction was started by adding TPNH. After temperature activation this lag disappeared (Fig. 8). The best measure of the effect of the temperature treatment appeared to be the difference in initial rates, although the initial rate catalysed by untreated mutant enzyme was difficult to assess and may, in fact, have been zero. In Figs. 5–7 rates measured over the first 90 sec. are shown, although this procedure probably overestimates true initial rates in the untreated or deactivated mutant enzyme.

It was found that the rapid increase in mutant-enzyme activity obtained by brief treatment at 35–50° was reversed, though relatively slowly, on returning the preparation to 21°. On repeating the heat treatment there was a second reversible activation (Fig. 5). In one experiment the cycle of operations was repeated three times, the degree of activation being the same on each occasion. Fig. 6 shows the time course of activation at different temperatures. At temperatures of 35° or more activation was very rapid, being complete in 90 sec. At 30° activation was very much slower and the final level of activity attained was somewhat lower than at 35° (although the activity may still have been slowly increasing at the end of the experiment). At 26° the rate of activation was very low. The data give the impression that the final activity obtained,

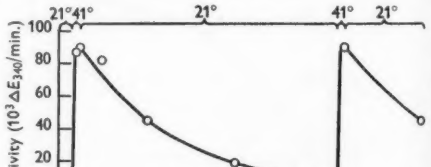

Fig. 5. Reversible temperature activation of the mutant enzyme. Enzyme from preparation 9 (Table 1) in 0·1 M phosphate buffer, pH 8·0 (0·4 mg. of protein/ml.), was kept in water baths at the temperatures indicated. Samples (1 ml.) were withdrawn at the times indicated and assayed in a system containing 0·08 M orthophosphate, pH 8·0, $3{\cdot}3 \times 10^{-3}$ N-$(NH_4)_2SO_4$, $6{\cdot}0 \times 10^{-3}$ M α-oxoglutarate and approx. $5{\cdot}4 \times 10^{-5}$ M TPNH after 3 min. at 21°. Reactions were started by addition of the TPNH. Untreated and deactivated samples gave accelerating reaction rates with initial rates close to zero; the mean rate over the first 90 sec. has been taken in each case.

as well as the rate of activation, is dependent on temperature in the range 25–35°, although this point is not fully established. The simplest explanation of these observations appeared to be that the mutant enzyme existed in two forms in temperature-dependent equilibrium, with an inactive form predominating at 21° and an active form predominating at 30°. Other more complicated explanations were not excluded however.

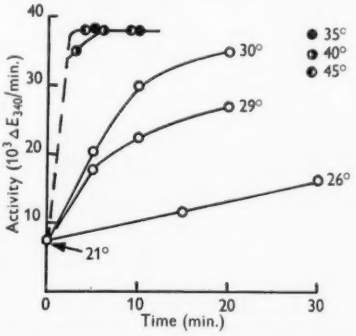

Fig. 6. Time course of activation of mutant enzyme at different temperatures. Enzyme from preparation 9 (kept frozen for 1 week) in 0·01 M phosphate buffer, pH 8·0 (0·24 mg. of protein/ml.). Samples (1 ml.) were withdrawn at the times indicated, equilibrated to 21° (room temp.) for 3 min., and immediately assayed as described under Fig. 5. The curve for 29° was obtained from a different experiment with the same enzyme (Fig. 7) by correcting for difference in enzyme concentration.

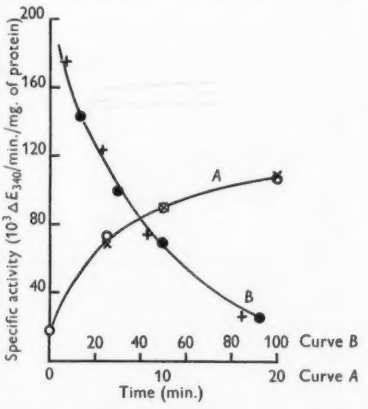

Fig. 7. Absence of effect of dilution on reversible temperature activation of mutant enzyme. Curve *A*: activation at 29° of prep. 9 (kept frozen for 9 days) in 0·1 M phosphate buffer, pH 8·0; 0·3 mg. of protein/ml. (×) and 0·15 mg. of protein/ml. (○). Curve *B*: decay of activity of the same preparation (0·4 mg. of protein/ml.) in the same buffer, after activation at 38° for 10 min. and (at time 0) transferring to 21° without further dilution (+) or diluted with an equal volume of buffer (●). Activity was assayed as described under Fig. 5.

It was conceivable either (*a*) that activation was due to the dissociation of the enzyme from an inhibitor and decay of activity to the recombination of the inhibitor with the enzyme, or (*b*) that activation was due to the combination of the enzyme with an activating impurity in the preparation, with decay in activity the reverse of this process. In (*a*) the rate of decay in activity (expressed as rate of change of specific activity) should decrease with increasing dilution of the enzyme preparation, and in (*b*) the same should apply to the rate of activation. To test these possibilities the effects of dilution on the rates of activation and decay in activity were investigated, 29° being selected as giving a suitably slow activation rate. Neither of the two possible dilution effects was found (Fig. 7), and it was concluded that a reversible change in the enzyme molecule itself was involved.

Effects of substrates on the activity of the mutant enzyme

The relation between activity of mutant enzyme (preparation 10, Table 1) and NH_4^+ ion concentration was found to be of the usual Michaelis type, with a Michaelis constant of close to 0·6 mM. In this experiment the enzyme was pre-incubated with α-oxoglutarate and TPNH to overcome the lag effect (see below). The relation between activity and α-oxoglutarate concentration was more complicated (Fig. 10), and will be discussed in relation to the lag effect already mentioned.

The lag in reaction rate, which was observed when the reaction catalysed by untreated mutant enzyme was started by the addition of TPNH, was not shortened by more prolonged incubation of the enzyme with α-oxoglutarate and ammonium salt before the start of the reaction. Nor was the lag altered appreciably by incubation of the enzyme with TPNH and ammonium salt, the addition of α-oxoglutarate being used to start the reaction. However, when the enzyme was incubated for 4·5 min. with TPNH and α-oxoglutarate and the reaction started by the addition of the ammonium salt, not only was the lag abolished but the maximum rate attained was considerably increased (Fig. 8). Fig. 9 shows that during incubation of the mutant enzyme with TPNH and α-oxoglutarate its activity increases up to a maximum achieved (under the conditions of this experiment) in about 6 min. An experiment similar to that illustrated in Fig. 8, but with wild-type enzyme, showed no such effect; the reaction rate was the same irrespective of the order in which the substrates were added.

The activating effect of α-oxoglutarate, in the presence of TPNH, on the mutant enzyme may help to explain the observations shown in Fig. 10. When the activity of untreated mutant enzyme was plotted against α-oxoglutarate concentration a sigmoid curve was obtained instead of the usual Michaelis relationship. The activity of the enzyme at the lowest α-oxoglutarate concentrations was almost negligible. After temperature activation of the enzyme a curve approximating to the Michaelis–Menten type was obtained, with a Michaelis constant of approximately 2 mM (rather lower than for the wild-type enzyme). It is noteworthy that the higher the α-oxoglutarate concentration, the less was the effect of temperature treatment. In a separate experiment it was found that mutant enzyme incubated with TPNH and 2×10^{-2}M α-oxoglutarate for 4·5 min. had as much as 70 % of the activity of the same preparation after temperature activation. It thus seems reasonable to suggest that the mutant enzyme can be converted into the active form either by temperature treatment or by incubation with saturating concentrations of α-oxoglutarate in the presence of TPNH. This hypothesis is further developed in the Discussion.

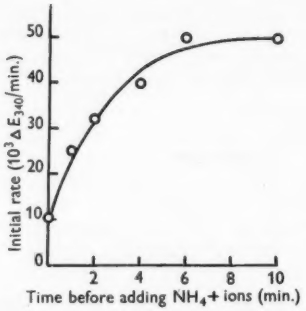

Fig. 9. Time course of substrate activation of mutant enzyme. Reaction mixtures with 0·09M orthophosphate, pH 8·0, 10×10^{-3}M α-oxoglutarate, $3{\cdot}3 \times 10^{-3}$N-$(NH_4)_2SO_4$, approx. $5{\cdot}3 \times 10^{-5}$M TPNH and 0·40 mg. of mutant protein from preparation 9 (Table 1), kept frozen for 3½ weeks. The ammonium salt (in 0·2 ml.) was added to the other components (2·8 ml.) at different times after mixing, except for the zero-time assay where the enzyme was added last. The non-pre-incubated enzyme gave an increasing reaction rate; the mean rate over the first 90 sec. is plotted.

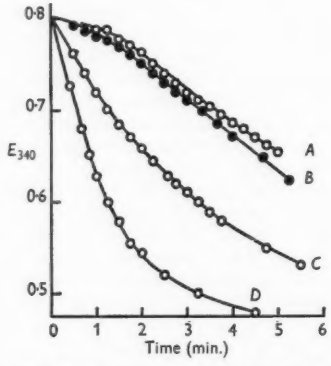

Fig. 8. Effect of pre-incubation with substrates on activity of mutant enzyme. Reaction mixtures with $6{\cdot}67 \times 10^{-3}$M α-oxoglutarate, $3{\cdot}3 \times 10^{-3}$N-$(NH_4)_2SO_4$, 0·09M orthophosphate, pH 8·0, approx. 6×10^{-5}M TPNH, and 1 mg. of protein of preparation 9 (Table 1) in 3 ml. Curve *A*: enzyme mixed with buffer, α-oxoglutarate and ammonium salt (2·8 ml.) 4 min. before addition of TPNH (0·2 ml.) at zero time. Curve *B*: enzyme mixed with buffer, TPNH and ammonium salt (2·9 ml.) 4 min. before the addition of α-oxoglutarate (0·1 ml.) at zero time. Curve *C*: enzyme mixed with buffer, TPNH and α-oxoglutarate (2·9 ml.) 4 min. before the addition of ammonium salt at zero time. Curve *D*: enzyme activated by 3 min. at 41°, returned to room temp. (3 min. at 21°), and treated as for curve *A*.

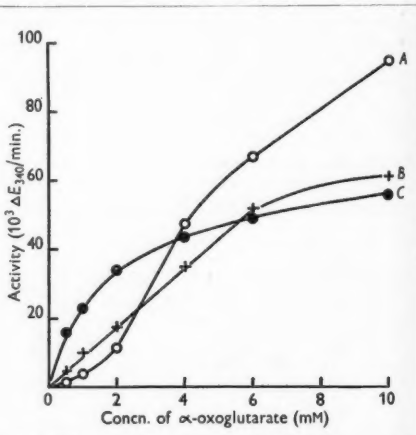

Fig. 10. Effect of α-oxoglutarate concentration on activity of mutant enzyme. Reaction mixtures with 0·09M orthophosphate, pH 8·0, $3{\cdot}3 \times 10^{-3}$N-$(NH_4)_2SO_4$, approx. $4{\cdot}7 \times 10^{-5}$M TPNH and (curve *A*) 0·64 mg. of protein from preparation 9 (Table 1); (curve *B*) 0·21 mg. of protein of the same preparation activated by 3 min. at 41°, or (curve *C*) 0·59 mg. of the same preparation activated by 6 min. at 38·5° after 3 weeks of frozen storage. Reactions were started by adding the ammonium salt (in 0·2 ml.) 4½ min. (curve *A*) or 2 min. (curve *B*) after mixing the other components, or (curve *C*) by adding the TPNH. The difference in maximum activity between curves *B* and *C* is attributed to ageing of the enzyme, and the difference in shape to the different order of addition of substrates (see text).

It seemed of interest to determine whether substrates had any effect on the rate of decay of activity of temperature-activated enzyme. α-Oxoglutarate alone, at a concentration of $1{\cdot}67 \times 10^{-2}$ M, was found to have no effect on the rate of decay of activity at 22°. The addition of TPNH, or TPNH plus ammonium salt, to temperature-activated enzyme caused a very much more rapid loss of activity than occurred in the absence of these substrates (Table 3). The activity was much more stable when both TPNH and α-oxoglutarate were present than when TPNH was added alone. These results are consistent with the observation, made in experiments on the effect of α-oxoglutarate concentration on the activity of temperature-activated mutant enzyme, that enzyme activity fell off rapidly with time in reaction mixtures containing TPNH and low α-oxoglutarate concentrations, but that this did not occur to the same extent at higher α-oxoglutarate concentrations. This probably explains the difference in shape of the two curves for activated enzyme shown in Fig. 10; unduly low activities were obtained where the enzyme had been briefly incubated with TPNH and low concentrations of α-oxoglutarate.

Table 3. *Effect of substrates on the rate of decay of activity of heat-activated mutant enzyme*

Mutant enzyme, preparation 9, 0·31 mg. of protein/ml. in 0·1 M phosphate, pH 8·0, was activated by 5 min. at 38°, and used at room temperature (21·5°) 3 min. later. Final concentrations in reaction mixtures: $3{\cdot}3 \times 10^{-3}$ N-$(NH_4)_2SO_4$, $6{\cdot}7 \times 10^{-3}$ M α-oxoglutarate, 0·08 M phosphate, pH 8·0, approx. 6×10^{-5} M TPNH, and 0·31 mg. of protein/3 ml.

Treatment of enzyme	Activity (ΔE_{340}/min. × 1000)
Mixed with NH_4^+ ions and α-oxoglutarate in 2·8 ml.; TPNH in 0·2 ml. added after 3½ min.	60
Mixed with TPNH and α-oxoglutarate in 2·8 ml.; NH_4^+ ions in 0·2 ml. added after 3½ min.	58
Mixed with TPNH and NH_4^+ ions in 2·8 ml.; α-oxoglutarate in 0·2 ml. added after 3½ min.	11, rising to 20
Mixed with buffer only in 2·4 ml.; other reactants in 0·6 ml. added after 5 min.*	45
Mixed with buffer and TPNH in 2·6 ml.; other reactants in 0·4 ml. added after 5 min.*	20, rising to 25

* Not closely comparable with the first three tests since the enzyme was one day older.

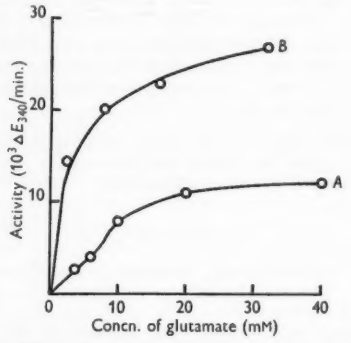

Fig. 11. Effect of glutamate concentration on activity of mutant enzyme. Reaction mixtures with 0·075 M orthophosphate, pH 8·0, approx. $1{\cdot}3 \times 10^{-4}$ M TPN and 0·2 mg. of protein of preparation 10 (Table 1). In curve *A* the enzyme was untreated and in curve *B* it was activated by 6 min. at 38·5°, cooled to 21° (room temp.) for 2 min., and used immediately. Reactions were started by the addition of the TPN. In curve *A* the reactions showed a slight initial lag; maximum rates are plotted.

A few experiments were made to determine the effect of glutamate concentration on the rate of reduction of TPN by mutant enzyme. It was found that in this case also there was an appreciable lag (after starting the reaction by addition of TPN) before the maximum rate of reduction was attained. This lag, which was not observed in similar experiments with the wild-type enzyme, was abolished, and the maximum rate was much increased, by temperature activation of the enzyme. The activated enzyme gave a concentration–activity curve for glutamate of the Michaelis–Menten type, with a Michaelis constant of about 2·5 mM (less than the value found for the wild-type enzyme). In the corresponding experiment with untreated enzyme the activities at low glutamate concentrations seemed unduly low (Fig. 11), and the Michaelis constant was apparently considerably higher than for the activated enzyme. These observations suggest that glutamate plus TPN, like α-oxoglutarate plus TPNH, tends to activate the enzyme, though the activation seems never to be complete in this case; even at the highest glutamate concentrations the activities of temperature-activated and untreated enzymes show no tendency to converge. It is possible that higher concentrations of TPN were required for complete activation, but this point has not been investigated.

DISCUSSION

It seems clear that the mutant enzyme must be qualitatively different from the wild-type enzyme, and that the mutant enzyme exists in two readily interconvertible forms.

While it is possible that temperature activation and substrate activation may be quite unrelated, it seems simpler to suppose that they represent two different ways of bringing about the same change in

the enzyme. This supposition is encouraged by the fact that at high α-oxoglutarate concentrations substrate activation tends to give nearly the same activity as temperature activation.

The following general hypothesis is offered to cover the two types of activation and also the anomalous relation between rate of reaction and α-oxoglutarate concentration. The mutant enzyme can exist in an inactive form *Ei*, or an active form *Ea*, the former predominating at 20° and the latter at 30° or higher. In the enzyme–TPNH complex, the position of the equilibrium at 20° again strongly favours the inactive form, and the attainment of this equilibrium is evidently more rapid than in the free enzyme. The TPNH–enzyme–α-oxoglutarate complex, however, is, at equilibrium, mainly in the active form at 20°, this implying that the affinity of *Ea*–TPNH for α-oxoglutarate must be much greater than that of *Ei*–TPNH. When untreated mutant enzyme is mixed at 20° with TPNH and α-oxoglutarate, the initial rate of activation will be proportional to the concentration of the TPNH–*Ei*–α-oxoglutarate complex, which, since the affinity of TPNH–*Ei* for α-oxoglutarate must be relatively very low, will be nearly proportional to α-oxoglutarate concentration over all but the upper part of the experimental concentration range. Thus the activity attained after a given period of incubation will be a function of α-oxoglutarate concentration (and possibly of TPNH concentration as well, though there is no evidence bearing on this point). Since the activity of the active form of the enzyme will itself be dependent on substrate concentration in the usual way (Fig. 10, curve *C*), the activity found will tend to increase with the square of the α-oxoglutarate concentration at low concentrations. Thus the sigmoid form of curve *A* (Fig. 10) seems explicable on the basis of the substrate-activation effect.

It would be surprising if such a difference in enzyme properties as exists between mutant and wild-type strains did not result in any difference in growth. In fact, though the mutant can scarcely be distinguished from wild type by growth experiments at 25°, it grows poorly on the usual minimal medium at 20° and is strongly stimulated by added α-amino acids at this temperature. The experiments are reported fully elsewhere (Fincham & Pateman, 1957). Thus there seems to be a good correlation between the *in vivo* and *in vitro* characteristics of the mutant.

From the point of view of physiological genetics the present case is of interest as an example of a qualitative change in a specific enzyme brought about by gene mutation. Such cases have hitherto been rare (but see Horowitz & Fling, 1953; Maas & Davis, 1952), although many mutations are known, both in *Neurospora* and in other micro-organisms, which have caused an apparently complete loss of an enzyme. With glutamic dehydrogenase of *N. crassa* a series of three alleles, corresponding to normal enzyme, altered enzyme, and apparent absence of enzyme, is now known (Fincham & Pateman, 1957).

SUMMARY

1. Glutamic acid dehydrogenase has been extracted and somewhat purified from wild-type *Neurospora crassa*, and some of its properties have been determined.

2. A mutant type, obtained secondarily by ultraviolet-light-induced mutation from a strain lacking detectable glutamic dehydrogenase, has been shown to produce a glutamic dehydrogenase different from the wild-type enzyme. The mutant enzyme has very little activity at 20°, but becomes active after a few minutes at 35–50°. This temperature activation was completely reversible; all the extra activity was lost after 2 hr. at 21°, but was quantitatively regained on repeated heat treatment. Experiments on the effect of dilution on activation and on loss of activity indicated that neither of these processes was due to a reaction with an impurity in the preparation.

3. Some activation of the mutant enzyme also resulted from incubation with substrates; a high concentration of α-oxoglutarate plus reduced triphosphopyridine nucleotide gave almost as much activation as did temperature treatment, whereas glutamate plus the oxidized coenzyme appeared to be less effective. The interpretation of these results is discussed.

4. The activity of the wild-type enzyme was quite unaffected by the procedures which increased the activity of the mutant enzyme. Experiments in which mixtures of wild-type and mutant enzymes were tested showed no interaction between the two types of preparation.

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Qualitative and Quantitative Changes Observed in the Free α-Amino Nitrogen Fraction of *Tenebrio molitor* Pupal Tissues during Metamorphosis

By D. S. P. PATTERSON
*Department of Biochemistry, University College London**

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An outstanding feature of the biochemistry of insects is the fact that a very high concentration of free amino acids has been found in the haemolymph of every insect so far investigated. (See Florkin, 1949; Buck, 1953.) This represents about two-thirds of the total free amino acids in the insect, and concentrations of from 20 to 100 times that of the total free α-amino nitrogen content of mammalian blood are generally found.

The chemistry of insect metamorphosis has attracted attention for at least 50 years. Needham (1929) made a comprehensive review of the information available in 1929, and since then more recent work has been summarized by Buck (1953) and Wigglesworth (1950, 1954). The variation in the titre of free amino acids during the metamorphosis of insects has been studied by Heller & Moklowska (1930) in *Deilephila*, by Florkin (1937) in *Bombyx mori* and by Agrell (1949) in *Calliphora*. In the work of Heller & Moklowska (1930) and Florkin (1937), the total free amino nitrogen content of insect blood was measured, and in Agrell's (1949) investigation changes in the concentrations of the free amino acids of pupal tissue breis were observed semi-quantitatively on paper chromatograms. In these three cases it was found that the level of total free amino nitrogen varied little during metamorphosis in spite of the tremendous morphological changes which holometabolous insects undergo at this period of their existence.

The present work describes the variations found in the free α-amino nitrogen content of the pupal tissues of another holometabolous insect, *Tenebrio molitor*, during metamorphosis at 24°, and an attempt is made to relate these findings with the observed changes in various other non-protein tissue fractions.

MATERIALS AND METHODS

Insects. Larvae of *Tenebrio molitor* were kept in a glass aquarium tank, the bottom of which was covered to a depth of 1–2 in. with a diet consisting of 2 parts by weight of bran, 1 part by weight of rolled oats and a supplement of 5% of dried yeast. A supply of water was also available to the insects in the form of a permanently damp pad of cotton wool. By careful daily inspection of the tank fresh pupae were identified and transferred to separate glass jars kept at 24°. The 'period of metamorphosis', which is defined for the present purposes as the time taken for a fresh pupa to become transformed into an adult insect at 24°, lasted 10 days. By making daily collections of fresh pupae it was ensured that samples of pupal tissue of any age were readily available from the beginning of the 10-day period.

Homogenates. All analyses were made on homogenates prepared from pupae with ice-cold water. Chilled pupae were killed by decapitation and homogenized with five times the weight of ice-cold water in a Potter–Elvehjem (1936) homogenizer. Fragments of cuticle were removed by straining through fine gauze.

Total free α-amino N. The ninhydrin–CO_2 titrimetric method of Van Slyke, MacFadyen & Hamilton (1941) was used to estimate the total free α-amino N in 70% (v/v final concn.) ethanolic extracts of pupal-tissue homogenates.

Ethanol was used as a deproteinizing agent because samples of the resulting protein-free filtrates were easily concentrated at comparatively low temperatures and were then suitable for amino acid chromatography. It was also found that the α-amino N contents of 70% ethanolic extracts of homogenates were only about 10% higher than the 1% picric acid extracts favoured by Hamilton & Van Slyke (1943).

Paper chromatography of free amino acids. Samples of the 70% ethanolic extracts obtained at daily intervals during metamorphosis were evaporated to dryness. The residues were freed from fat by extraction with ether and then dissolved in small volumes of water. Samples of the aqueous extracts each approximately equivalent to the same dry weight of tissue were then applied from capillary pipettes as compact spots to the corners of 20 cm. × 20 cm. sheets of Whatman no. 1 paper, and the series of two-dimensional ascending paper chromatograms were developed simultaneously (Datta, Dent & Harris, 1950), first with 80% phenol–ammonia and then 90% Methyl Cellosolve (2-methoxyethanol; Bender, 1951) as irrigating solvents.

Citrate. The microcolorimetric method of Weil-Malherbe & Bone (1949) was used with the modification that the final colour production was carried out by extracting the light petroleum solution of pentabromoacetone with a solution of thiourea buffered with borax (Natelson, Pincus & Lugodvy, 1948). Citrate was estimated in 5% (w/v final concn.) trichloroacetic acid extracts of the tissue homogenates.

Uric acid. Tenfold dilutions of the 1/5 tissue homogenates were deproteinized by suspending 1 ml. in 5 ml. of water

* Present address: Biochemistry Department, Ministry of Agriculture, Fisheries and Food, Veterinary Laboratory, Weybridge, Surrey.

and then adding 1 ml. each of 10% (w/v) sodium tungstate solution and 0·67 N-H_2SO_4. After making the volume up to 10 ml. and mixing thoroughly, the filtrate finally obtained was used for the estimation of uric acid by the Dresel & Moyle (1950) modification of the method of Brown (1945).

Nucleic acids. Protein and nucleic acids were first precipitated from samples of homogenates by ice-cold 5% (w/v final concn.) trichloroacetic acid. The precipitate obtained by centrifuging was allowed to drain thoroughly, washed with cold 5% trichloroacetic acid and then extracted with 5% trichloroacetic acid at 90° for 20 min. This extract was then used for the colorimetric estimations of deoxyribose nucleic acids (DNA) and ribosenucleic acids (RNA).

DNA was estimated in the trichloroacetic acid extract by the diphenylamine reaction of Dische (1930, 1955).

RNA was estimated by the Mejbaum (1939) modification of the Bial orcinol reaction for pentoses (see also Dische, 1955).

By preparing calibration curves for DNA and RNA from nucleic acid samples of known phosphorus content, it was possible to express results in terms of either nucleic acid or nucleic acid phosphorus content.

RESULTS

The total free α-amino N content of *Tenebrio* pupal tissue was determined in 70% ethanolic extracts of 1:5 homogenates. Since it is generally found that the free amino acid content of insect blood constitutes between 50 and 85% of the total non-protein nitrogen (Buck, 1953), the measurement of total free α-amino N was taken to be a measure of the 'pool' of free amino acids in *Tenebrio* pupal tissues. Two or three samples of homogenate were analysed in duplicate at the different stages of metamorphosis (in the present context, a stage is equivalent to the pupal age in days at 24°).

The average values obtained are given in Table 1, and it is seen that the free α-amino N fraction represented between 2·6 and 4·6% of the total N content of the homogenate. After an initial sharp decrease in the total free α-amino N, the level remained approximately constant from about day 3 to day 7. Subsequently and at about the time of ecdysis (day 10) the free amino acid content tended to rise once more. This is essentially the same kind of result that Florkin (1937) obtained when he measured the variation in total amino N in the blood of *Bombyx mori* pupae with a modification of Folin's colorimetric method. That is to say, at least in the holometabolous insects *Bombyx* and *Tenebrio*, there is a free amino acid 'pool' of approximately constant size during a considerable part of metamorphosis. At the beginning and end of pupal life, however, certain fluctuations are noticed in both these insects and also in *Deilephila* (Heller, 1924), where a decrease in free amino N was observed at the end of metamorphosis.

One-dimensional paper chromatography of the free amino acids in breis of *Calliphora* was described by Agrell (1949). Here a semi-quantitative evaluation of the variation in the quantities of free amino acids was made and it was found that, apart from a fall in concentration at about one-fifth of the way and another at about three-quarters of the way through metamorphosis, there was an approximately constant level of free amino acids throughout pupal life. During the present work on *Tenebrio* homogenates a semi-quantitative investigation was made with two-dimensional paper chromatography.

After development of the chromatograms and after ensuring complete removal of irrigating solvents, the paper sheets were uniformly sprayed with 0·5% solution of ninhydrin in butanol, and the amino acid spots were revealed by heating in an electric oven at 110° for 10 min. The amino acid spots were identified by comparison with a 'map of spots' which had been obtained when standard solutions of known amino acids were chromatographed both singly and as mixtures. The two-dimensional array of spots was similar to that described by Dent (1948), except that R_f values of amino acids in the direction of the development with 90% Cellosolve were found to be generally greater (cf. Bender, 1951).

Table 1. *Some changes in the chemical composition of* Tenebrio *pupal tissues during metamorphosis at* 24°

Stage of metamorphosis (days)	Average total free α-amino N (μg./pupa)	Average citrate content (μg./pupa)	Ratio α-amino N: citrate	α-Amino N as % of total N (average)	Average uric acid N content (μg./pupa)	Average RNA content (μg./pupa)	Average DNA content (μg./pupa)	Ratio RNA:DNA
Pupa 0	300	109	2·8	4·6	139	494	107	4·6
1	219	146	1·5	3·1	145	527	113	4·7
2	208	157	1·3	2·6	172	408	158	2·6
3	242	184	1·3	3·0	171	387	136	2·8
4	258	172	1·5	3·0	174	328	133	2·5
5	256	160	1·6	3·2	184	305	163	1·9
6	263	158	1·7	3·3	178	354	172	2·1
7	267	144	1·9	3·5	170	319	159	2·0
8	333	147	2·3	3·1	198	356	167	2·1
9	267	105	2·5	3·2	216	322	118	2·7
10	354	118	3·0	4·3	252	414	152	2·7
Adult 1	—	—	—	4·2	—	569	131	4·3

By comparing twelve chromatograms representing samples of tissue obtained at each day of pupal life and on the first day of adult life, at the same time in reflected daylight, a record was made in arbitrary units of the variations observed in the concentrations of the individual amino acids comprising the free amino-acid 'pool'. No attempt was made to relate concentrations of amino acids to one another. The figures given in Table 2 show how the different amino acids were found to vary in concentration during metamorphosis with this procedure. Since the free amino acids were not completely resolved on these chromatograms, two of the twelve spots obtained represent mixtures of two or three amino acids, and variations in concentration of these composite spots may not be attributed to changes in concentration of any specific amino acids.

Certain striking changes in the qualitative composition of the free amino acid pool were seen during the metamorphosis of *Tenebrio*. Aspartic acid, glycine, glutamic acid, proline and glutamine showed a general downward trend. One or both of the components of the arginine–lysine spots and one, two or all of the amino acids valine, methionine and tryptophan also decreased in concentration. Tyrosine gradually built up to a very high concentration, and was probably mobilized as a substrate for synthesis of cuticle melanin pigment as reported by Fraenkel & Rudall (1947) in the pupa of *Sarcophaga*. Concentrations of alanine and the leucines remained almost unchanged, whereas the hydroxy-amino acids serine and threonine increased in concentration at first and then began to decrease from day 5 or 6 onwards. A number of amino acids were seen to decrease in concentration just before ecdysis (days 9 and 10), but afterwards increased concentrations were seen in most cases. This corresponded with the terminal increase observed in the total free α-amino N concentration.

Until recently, the only citrate analysis reported in insects was due to Levenbook (1950). The blood of the larva of *Gastrophilus intestinalis* was examined exhaustively by this author and found to contain 45 mg. of citrate/100 ml. This insect, like all others so far investigated, also possessed a high free amino N concentration (93·8 mg./100 ml.). Examination of the blood of *Rhodnius prolixus* and *Tenebrio molitor* yielded similar results (Patterson, 1956). The concentration of citrate in the haemolymph of *Rhodnius* was found to be 44 mg./100 ml. and in *Tenebrio* 97 mg./100 ml. The corresponding free α-amino N concentrations were 75·3 and 180·5 mg./100 ml.

At first sight it appeared that there might be a direct relationship between the accumulation of the free amino acid and citrate. In all three insects for which figures were available the ratio of total α-amino N to citrate concentration was between 2·1

Table 2. *Daily semi-quantitative analyses of the free amino acids in* Tenebrio *pupal tissues during metamorphosis at* 24°

Concentrations of individual amino acids and mixed spots are expressed in arbitrary units. Tr., trace detected.

Stage of metamorphosis (days)	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	Tyrosine	Glutamine	Arginine + lysine	Proline	Valine + methionine + tryptophan	Leucines
Pupa 0	1	3	1	5	1	1	2	3	4	2	3	3
Pupa 1	1	3	1	3	1	1	2	2	4	2	3	3
Pupa 2	1	3	2	3	2	1	1	3	4	2	3	2
Pupa 3	1	3	2	2	2	1	1	2	4	2	3	2
Pupa 4	0	3	2	2	2	1	1	2	4	2	3	2
Pupa 5	0	3	3	2	2	1	1	2	4	2	2	2
Pupa 6	0	3	3	3	1	1	2	1	3	1	1	2
Pupa 7	0	2	2	2	1	1	2	1	3	1	1	2
Pupa 8	Tr.	2	2	2	1	1	3	1	3	1	1	2
Pupa 9	0	2	1	1	0	1	4	1	2	1	1	2
Pupa 10	0	1	1	1	0	1	4	1	2	1	1	2
Adult 1	Tr.	2	1	3	Tr.	1	5	1	2	2	3	2

and 2·4. And in order to test this possibility, citrate was estimated in duplicate on two to four samples of *Tenebrio* pupal tissue taken at different stages of development. Total free α-amino N was also estimated on the same tissue samples. The ratio of total free α-amino N:citrate was calculated for each sample of tissue and average values obtained for the daily stages of metamorphosis are given in Table 1; it is concluded from these figures that there is no evidence to suggest that a direct relationship obtains between the two concentrative processes.

Two major pathways for the utilization of amino acids are the synthesis of proteins from amino acids and the deamination of amino acids before utilization either for the synthesis of non-protein molecules or for eventual oxidation to yield energy. Deamination may give rise either to ammonia or to a transferable amino group. The former product is detoxicated in animals with a restricted supply of water, and in insects it is well known that uric acid is the final end-product of this process (Baldwin, 1940; Florkin, 1949). In the *Tenebrio* pupa, which is an enclosed system from the point of view of nitrogen metabolism (i.e. it neither feeds nor excretes during the whole period of metamorphosis), and in the absence of further breakdown of uric acid (D. S. P. Patterson, unpublished results) this substance accumulates in the pupal tissues. The rate of uric acid accumulation must be largely dependent upon the rate of deamination and its measurement may be legitimately used as an approximate measure of deaminase activity during given phases of metamorphosis.

The uric acid content of samples of *Tenebrio* pupal tissue homogenates was estimated at different stages of metamorphosis and the average figures obtained (four or five samples at each stage) are recorded in Table 1. From the results it appeared that the rate of accumulation went in three phases during metamorphosis. The first phase ended at day 2, the second at day 7 and the third ended with ecdysis. Since the average uric acid content of a *Tenebrio* larva was found to be about 75 μg., the total amount of uric acid in each insect is doubled during the first 2 days of pupal life. This, then, is a period of very rapid deamination. From day 2 to day 7 the average uric acid content of the pupa is nearly constant and consequently deamination must proceed at a minimal rate. Thereafter a second period of rapid deamination is observed, the level of uric acid being increased by about another 50% during the last 3 days.

There is a well-known correlation between protein synthetic activity and an increase in the RNA content of tissues (Brachet, 1955). Consequently, provided that the number of cells remains unchanged in samples of insect tissue taken at different stages of development, an observed increase in RNA at any particular stage would probably correspond with a period of pronounced protein synthesis. In the absence of cytological techniques for counting the nuclei present in samples, it is permissible to assume, provided that the DNA content of tissue samples is constant, that an increase in the RNA:DNA ratio (which represents a true increase per unit of nuclear material) would have the same significance.

Therefore RNA and DNA estimations were made on from two to five samples of homogenates of pupal tissue taken at different stages of metamorphosis. The ratio of RNA content to DNA content was calculated and expressed in terms of nucleic acid phosphorus. Average values quoted in Table 1 represent the averages of the various values of this ratio obtained for individual tissue samples at the same stage of development. Whereas the average DNA content appeared to be evenly distributed about a mean value of 140 μg./pupa, the average RNA fell from about 510 μg./pupa at the beginning of pupal life to about 330 μg./pupa during days 4 to 6. Thereafter, it rose to about 570 μg. of RNA/insect in the first day of adult life. The ratio RNA:DNA also exhibited a U-shaped variation during metamorphosis, so that taking into account the approximately constant concentration of DNA (a measure of nuclear material) it appears that at the beginning and at the end of metamorphosis there was a high level of RNA per cell, and therefore these two periods of development might well be associated with intensive protein synthetic activity.

DISCUSSION

During the metamorphosis of holometabolous insects considerable morphological changes take place, and proteolysis of larval proteins and subsequent or even concurrent synthesis of pupal and adult tissue protein must be expected. With the continuous re-utilization of amino acid molecules derived in the first place from larval proteins it might be anticipated that rather considerable fluctuations in the level of total free α-amino nitrogen would be observed. In fact, it has been shown that for a considerable part of the metamorphosis period of *Tenebrio molitor* the total free α-amino nitrogen content of pupal tissue is approximately constant. Essentially the same observation was made by Florkin (1937), who analysed the blood of *Bombyx mori* pupae, and by Heller (1924) and Heller & Moklowska (1930) who examined *Deilephila* pupal tissues.

Agrell (1949) made a semi-quantitative investigation of the free amino acids in *Calliphora* pupal breis and also observed the approximately constant size of the free amino acid 'pool'. While considering the concentration of free amino acids to be maintained

by a balance of protein synthesis and protein breakdown, Agrell suggested that quantities in excess of an equilibrium titre of free α-amino nitrogen were oxidized, especially at the beginning and at the end of metamorphosis. These periods of more intensive amino acid oxidation corresponded with the peaks of the U-shaped oxygen utilization and various amino acid dehydrogenase-activity curves. The present results which indicate a maximal deaminase activity in *Tenebrio* pupal tissues at the same periods of pupal life are in some agreement with Agrell's observations, although the approximately constant level of α-amino nitrogen actually corresponded with a period of minimal deamination (day 3 to day 7).

The initial drop observed in the total free α-amino nitrogen content of pupal tissue coincided understandably with a phase of rapid deaminase activity, whereas the final increase in the free α-amino nitrogen titre and the general increase in concentrations of individual amino acids separated by paper chromatography also occurred at a time when the deamination was intensive. This apparent incompatability between the two sets of data may, however, be understood once it is realized that towards ecdysis, amino acid metabolism is complicated by the fact that a large proportion of the protein of the pupal cuticle is actually absorbed by the insect (Evans, 1938). In this event, cuticle protein, probably absorbed as amino acids, may easily be in excess of the capacity of the deaminating enzymes, and therefore in spite of the observed rapid rate of accumulation of uric acid at this period the total free α-amino nitrogen level tends to rise.

The 'pool' of free amino acids represents a balance between protein breakdown and amino acid utilization, but mechanisms which control the extent to which amino acids accumulate are not understood. The accumulation of high concentrations of amino acids in certain tissues may possibly be associated with high protein synthetic activity. Thus it has been observed by Van Slyke & Meyer (1913) that mammalian hepatic tissue has a greater ability to concentrate amino acids than skeletal muscle. (Dog muscle saturation value was about 70 mg./100 g. Liver was saturated at about 157 mg./100 g.) Awapara (1952) pointed out that in the ventral prostate of the dog, an elevated level of free amino acids was accompanied by an increased citric acid content and thought it possible that the two concentrative processes were interrelated.

The elevated level of citrate in *Tenebrio* pupal tissue was not found to be directly related to the accumulation of free α-amino nitrogen. There was, however, a noticeable variation in tissue-citrate concentrations, and the maximum citrate accumulation occurred at about the time of least oxygen utilization. In fact there has been shown to be an approximately linear relationship between tissue-citrate concentration and the rate of oxygen utilization by live pupae (Patterson, 1956). This led to the speculation that during the metamorphosis of *Tenebrio molitor* the reactions of the tricarboxylic acid cycle functioned more or less efficiently according to whether relatively low or very high concentrations of citrate were to be found in the tissues.

Since certain keto acids and especially α-oxoglutarate are synthesized by means of the reactions of the tricarboxylic acid cycle it would not be unreasonable to suppose that at a period of the cycle's least efficiency (at about day 3), deamination, probably initiated by α-amino group transfer to α-oxoglutarate, would be carried out at a slow rate. In fact, at this phase of pupal life, the rate of accumulation of uric acid (the end-product of deamination) was minimal. Moreover, a decrease in the concentrations of certain individual amino acids such as aspartic acid, glutamic acid, glutamine, threonine and proline was more noticeable (Table 2) as the concentration of citrate fell from the maximum value, i.e. from day 4 onwards.

The variations in the RNA:DNA ratio which have been seen during the pupal period have not been observed before in insect development. In 1952, however, Agrell reported complementary variations in the RNA phosphorus and DNA phosphorus contents of *Calliphora* pupae during metamorphosis and deduced that the two nucleic acids were interconverted by this insect. The ratios RNA P:DNA P were not recorded in this paper by Agrell (1952), but calculations made from his data show that there was also a similar U-shaped variation in this case of insect development. The actual figures so obtained for RNA P:DNA P are somewhat low compared with those recorded in mammalian tissues, and in the present work on *Tenebrio* pupal tissue (Table 3), but they may have been influenced by his use of the method of Ogur & Rosen (1950) for estimating nucleic acids. Davidson (1953) has claimed that results obtained by this method are misleading owing to contamination by inorganic phosphate unless prior ionophoretic separation of the soluble nucleotides is undertaken.

From the beginning of pupal life the value of RNA:DNA was seen to fall to a fairly uniform value of about 2·0, which was estimated on tissue samples from day 5 to day 8, the value increasing again thereafter. It was supposed, then, that at the beginning of the pupal period protein synthesis was more rapid than elsewhere during metamorphosis. The increasing value obtained for the ratio at about the time of the emergence of the adult was probably associated with protein synthesis in the new adult cuticle. Both phases of increased protein synthetic activity occurred at times when the titre of free amino acids was relatively high and clearly this is an advantageous situation.

Table 3. *Ratio of RNA phosphorus: DNA phosphorus in various animal tissues*

Tissues	RNA P:DNA P	Source
Calliphora (pupae)		
Beginning of pupal life	0·71	Agrell (1952)
Middle of pupal life	0·30	
At ecdysis	0·60	
Tenebrio molitor (pupae)		
Day 0	5·09	Present work
Day 5	2·14	
Day 10	3·02	
Tenebrio molitor (young adult)	4·83	
Rat liver	4·0	Davidson (1953)
Cat liver	2·3	
Human liver	3·0	
Cat pancreas	3·4	
Rat mammary gland (lactating)	2·7	T. F. Slater (private communication)

The period of intensive protein synthesis which is indicated at the very beginning of pupal life (RNA:DNA = about 4·6 at day 0 and day 1) is probably associated with the formation of adult structures within the pupa, which has been held by Dobzhansky & Paulson (1935) to be often completed in *Drosophila* before the period of minimum oxygen utilization. If this is a general feature of holometabolous insect development it means that the 'metamorphosis' hitherto referred to in this work is, in fact, largely the development of the adult within the pupal skin, the actual metamorphosis or transformation of larval structures having been undergone predominantly in the pre-pupal stage.

SUMMARY

1. The total free α-amino nitrogen content of *Tenebrio molitor* pupal tissues was measured during metamorphosis. For a considerable period (5 days), the level of α-amino nitrogen was approximately constant.

2. A decrease in the free amino acid titre observed at the beginning of metamorphosis is associated with high deaminase activity, and the increase which was seen at about the time of ecdysis is probably a result of the absorption of pupal cuticle protein.

3. Semi-quantitative examination of the free amino acids by means of two-dimensional chromatography generally reflected the changes in total free α-amino nitrogen.

4. The rate of deamination measured by the rate of accumulation of the ultimate nitrogenous end-product uric acid in the pupal tissues was noticed to be greatest at the very beginning of the metamorphosis, constant during the middle and elevated towards the end of pupal life.

5. There was no evidence for the accumulation of citrate in *Tenebrio* pupal tissues being directly related to synchronous accumulation of free amino acids.

6. The ratio RNA:DNA decreased after the beginning of the pupal period and increased towards its end. The maxima were thought to correspond with intensive protein synthetic activity during the formation of adult tissues and when the adult cuticle protein was being synthesized.

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The Effects of Phosphates, Arsenates and Nucleotides on L-Amino Acid Decarboxylases

By L. V. EGGLESTON
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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Gale & Epps (1944) and Gale (1945) observed that the lysine decarboxylase activity of *Escherichia coli* '86' was increased by inorganic phosphate (0·1–0·5M), and that the decarboxylation of ornithine by *Clostridium septicum* was more rapid in phosphate buffers than in citrate buffers of comparable pH. Krebs, Eggleston & Knivett (1955) recently confirmed that the decarboxylation of L-ornithine and L-lysine by *Esch. coli* was accelerated by the addition of phosphate at pH 6·5–6·8. The present paper is concerned with a more detailed study of the effects of inorganic and organic phosphates and related substances on the activity of amino acid decarboxylases.

EXPERIMENTAL

Materials

Buffers. The following buffers were used: phosphate, made from molar stock solutions of Na_2HPO_4 and NaH_2PO_4; arsenate, made from 0·5M-Na_2HAsO_4 and N-HCl; citrate made from 0·2M sodium citrate and N-HCl or N-NaOH; tris, made from M aminotrishydroxymethylmethane and N-HCl; acetate, made from sodium acetate and acetic acid; acetate–veronal (Michaelis, 1931), and tris–maleate (Gomori, 1955). A glass electrode and a Pye pH meter were used to measure the pH of these buffers after dilution to the concentrations used in experiments. The additions of enzyme and substrates had little or no effect on the pH.

Nucleotides. Adenosine triphosphate (ATP) was prepared in this laboratory by Mr R. Hems. The barium salt from rabbit muscle (LePage, 1949) was dissolved in 0·1N-HCl and passed through a column of Amberlite resin IR-120 (H) in the H^+ form. The free acid was neutralized with NaOH and concentrated by freeze-drying. Analysis by the method of Krebs & Hems (1953) showed it to be at least 98% ATP, with a trace of adenosine diphosphate (ADP), and no other detectable phosphate impurity. In some experiments two commercial samples of ATP were used; analysis showed them to be mixtures of ATP and ADP (about equal quantities) with about 15% of other impurities including inorganic phosphate, adenosine 5′-phosphate (AMP-5′) and some other unidentified organic phosphates.

Barium ADP was obtained from Schwarz Laboratories Inc. It was found to contain less than 5% ATP.

Muscle adenylic acid (AMP-5′) from Roche Products Ltd., and yeast adenylic acid from L. Light and Co. Ltd., were both found to be 98–100% pure when examined by the chromatographic techniques of Krebs & Hems (1953).

Adenosine 2′-phosphate (AMP-2′) and adenosine 3′-phosphate (AMP-3′) were obtained from L. Light and Co. Ltd. Examination of these compounds by the chromatographic method of Carter (1950) showed no phosphate impurities. Guanylic, uridylic and cytidylic acids, which are known to be mixtures of the 2′- and 3′-phosphates, were also obtained from L. Light and Co. Ltd.

Inosine triphosphate and inosine 5′-phosphate from Sigma Chemical Co. showed no phosphate impurities when tested by the method of Krebs & Hems (1953).

Flavin mononucleotide was a gift from the Sigma Chemical Co.

Other compounds. Barium ribose 5-phosphate was prepared according to Long (1955). Chromatographic analysis according to Eggleston (1954) revealed traces of AMP-5′, but no other impurity. For use it was dissolved in dilute HCl and a calculated amount of Na_2SO_4 was added to precipitate the barium; after centrifuging, the supernatant solution was adjusted to pH 6·8 with NaOH. Glucose 1-phosphate, glucose 6-phosphate and sodium arsonoacetate ($Na_2O_3.AsCH_2.CO_2Na$) were prepared by Mr D. H. Williamson. Other organic arsenicals were obtained from British Drug Houses Ltd. Organophosphorus insecticides were obtained

from Dr W. N. Aldridge and from Albright and Wilson Ltd. These included: di*iso*propylphosphorofluoridate, diethyl *p*-nitrophenyl phosphate (E 600; paraoxon), octamethylpyrophosphoramide (schradan, OMPA), dimethyl *p*-nitrophenyl phosphorothioate (parathion), tetraethyl pyrophosphate and dimethyldichlorovinyl phosphate. Carbamyl phosphate was prepared by Dr M. Shilo according to Jones, Spector & Lipmann (1955). Pyridoxal phosphate was supplied by Merck and Co. Inc., Rahway, New Jersey, U.S.A.

Micro-organisms. Clostridium welchii S.R. 12 and seven strains of *Escherichia coli* were examined. These included: *Esch. coli* 'F' (*Bact. coli*), a laboratory strain from the Middlesex Hospital, used by Fildes (1940) and by Woods (1940); *Esch. coli* 'T', a laboratory strain isolated in Professor D. D. Woods's laboratory; *Esch. coli* '4', a laboratory strain originating from Department of Biochemistry, University of Sydney, Australia, used by Lascelles & Still (1946); *Esch. coli* var. *communior*, N.C.T.C. no. 86; *Esch. coli* N.C.I.B. no. 8114, American Type culture collection no. 9723; *Esch. coli* 'M/48', origin unknown. All the above were supplied by Professor D. D. Woods. Dr V. A. Knivett supplied *Esch. coli* N.C.I.B. no. 8571 (Knivett, 1954).

Methods

Cl. welchii was grown as described by Krebs (1948). The *Esch. coli* strains were grown in 1 l. spherical, flat-bottomed flasks filled to the neck with a liquid medium containing 1% Lab-lemco (Oxo Ltd.), 0·5% yeast extract (Difco), 0·5% NaCl, 2% Tryptone (a pancreatic digest of casein, Oxo Ltd.) and 2% glucose (sterilized separately), all w/v. After inoculating the flasks with subcultures which had been grown overnight, the flasks were incubated at 27° for 24 hr. During this time the pH fell from about 7 to about 5. The cells were collected by centrifuging, washed twice with water, and stored at 0° or −15° as suspensions in water containing about 30 mg. dry wt. of cells/ml. Cell-free extracts were prepared in the Hughes press (Hughes, 1951) at −25° to −15°, or by shaking 10 ml. suspensions of whole cells with 10 ml. of Ballotini beads no. 14 (Chance Bros., Birmingham) in a Mickle disintegrator (Mickle, 1948) at 50 cyc./sec. for 30 min. at 0°, followed by centrifuging and washing of the sediment with small volumes of water. These extracts were in some instances dialysed against distilled water at 2° overnight, or were re-centrifuged in a high-speed head at 16 300 *g* to obtain enzymes in clear solution. Acetone–ether dried powders were prepared according to Gale & Epps (1944).

Extracts were made of carrot, vegetable marrow and melons by using a 'Braun' separator (Max Braun Commercial, Frankfurt a. M., Germany). In this instrument the tissue is grated and the liquid fraction is separated from insoluble particles by centrifugal forces. The extracts were filtered through a Jena sintered-glass funnel (grade 3G3) without suction (see Schales, Mims & Schales, 1946). The filtrate was stored at −15°.

Brains from pigeons and rats, and rat livers, were ground with 1 or 2 vol. of water in a mortar, or were homogenized in a stainless-steel Potter–Elvehjem homogenizer. Fresh suspensions were examined immediately wherever possible, as they lost 40–50% of their decarboxylating activity when stored for 1 day at −15°.

The decarboxylation of L-amino acids was examined in Warburg manometers in an atmosphere of N_2. Preliminary experiments (on the bacterial decarboxylation of arginine) were carried out at 40°, but as the bacterial decarboxylases are known to be rapidly inactivated at this temperature (Gale, 1940) most of the subsequent work on bacterial and plant enzymes was done at 25°. In experiments with animal tissues the temperature was 35°.

In experiments buffered at pH 6·3–6·8 a correction was made for the bound CO_2 according to Johnson (1951). Above pH 6·8 this procedure is not sufficiently accurate and in this pH range Warburg vessels with two side arms were used for the determination of the total CO_2 production, substrate being placed in one side arm, 0·5 ml. of 10% (v/v) H_2SO_4 in the other. The addition of the acid at the end of the experiment gave the value for bound CO_2. In some experiments the results are expressed as Q_{CO_2} (µl. of CO_2/mg. dry wt. of enzyme/hr.).

Paper chromatography of nucleotides was carried out on trichloroacetic acid filtrates according to Krebs & Hems (1953) and Carter (1950). For the chromatography of amino acids and amines the second solvent of Krebs & Hems was used (*iso*butyric acid–ammonia soln.–water) or phenol saturated with water at 18°. After a descending flow for 17–20 hr. at 22° a ninhydrin spray was used to detect amino acids and amines.

Ammonia was determined in trichloroacetic acid filtrates by distillation in Conway units followed by nesslerization. Inorganic phosphate was determined by the method of Berenblum & Chain (1938), as modified by Bartley (1953).

RESULTS

Bacterial decarboxylases

Permeability of cells. Preliminary experiments with whole cells suggested that access of L-arginine and nucleotides to the enzymes is a factor controlling reaction rates. The rate of decarboxylation of arginine by *Esch. coli* 'F' at pH 6·8 was examined under the following conditions: (*a*) whole cells which had been stored at 0°; (*b*) whole cells stored at −15°; (*c*) cell-free extracts prepared in the Mickle disintegrator (Table 1). In the absence of added nucleotide the liberation of CO_2 was very low in the whole cells; it was five times higher in the cells damaged by deep-freezing, and ten times higher in the cell-free extract. In the cells stored at

Table 1. *Effects of ATP, ADP and AMP-5′ on arginine decarboxylation in whole cells, damaged cells, and cell-free extracts of* Esch. coli *'F'*

Whole cells (7·7 mg. dry wt.) were stored at 2° or at −15°, or 4·2 mg. dry wt. of cell-free extract was stored at −15°; 0·025 M phosphate buffer, pH 6·8; 0·05 M L-arginine; 0·01 M nucleotides; final vol. 2 ml. Gas phase, N_2. Temp., 25°.

Additions	Q_{CO_2} for 0–30 min. period		
	Whole cells stored at 2°	Whole cells stored at −15°	Cell-free extract
—	4·6	20·7	49·0
ATP	4·4	31·2	59·0
ADP	5·5	34·2	95·2
AMP-5′	10·5	30·9	102·4

Table 2. *Effect of Cetavlon on arginine decarboxylation in whole cells and cell-free extract of* Esch. coli '*F*'

Expt. 1: 11·9 mg. dry wt. of whole cells. Expt. 2: 1·68 mg. dry wt. of cell-free extract. Phosphate buffer (0·025 M, pH 6·8); 0·025 M L-arginine; final vol. 2 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Cetavlon added (M)	Q_{CO_2} for 0–30 min. period
1	0	6·6
	5×10^{-5}	7·7
	5×10^{-4}	30·2
	5×10^{-3}	30·6
2	0	40·5
	5×10^{-4}	38·1

2°, 0·01 M AMP-5′ caused a doubling in the rate of decarboxylation; 0·01 M ADP caused an increase of about 20 % and 0·01 M ATP was ineffective. All three nucleotides gave approximately equal increases in rate (of about 50 %) with cells stored at −15°. In cell-free extracts, AMP-5′ was again the most effective stimulant, giving 105 % increase, whereas ADP and ATP increased the rate by 86 and 22 % respectively.

Cetavlon (cetyltrimethylammonium bromide), which accelerates the decarboxylation of glutamic acid in whole cells and extracts of *Cl. welchii* (Krebs, 1948; Hughes, 1949, 1950), greatly stimulated the decarboxylation of arginine in whole cells of *Esch. coli* but not in cell-free extracts (Table 2). This suggests that the effect of Cetavlon is due to an alteration of cell permeability. Most of the subsequent experiments were made on whole cells stored at 0°, to which Cetavlon (5×10^{-4} M) had been added just before the start of the incubation.

Whole cells stored at 0° showed little decline in decarboxylating activity over periods up to 4 weeks, whereas repeated deep-freezing and thawing of cells or cell-free extracts sometimes caused considerable losses in activity. These losses were rather inconsistent, varying with the organism used and the enzyme being studied.

Effects of phosphate, arsenate and adenosine 5′-phosphate. The seven strains of *Esch. coli* and *Cl. welchii* S.R. 12 were examined for the effects of 0·01 M-phosphate, arsenate and AMP-5′ on various amino acid decarboxylase activities at pH 6·4–6·8. As shown in Table 3 the decarboxylation of arginine, ornithine and lysine by the various strains of *Esch. coli* was accelerated in all cases by AMP-5′. The increases were particularly large in the cases of arginine decarboxylation by strains 'F', '4' and '8114' (+154, +193 and +149 % respectively). The largest increase caused by AMP-5′ was found during the decarboxylation of lysine by strain '4' (+255 %). In the decarboxylation of glutamate by *Cl. welchii*, there was no major effect during the first 30 min., but AMP-5′ caused a definite increase in rate in the 30–60 and 60–90 min. periods (+85 and +188 % respectively).

Appreciable increases in rate were caused by inorganic phosphate and arsenate but there were considerable strain differences. Major increases occurred in six of the fourteen combinations of organism plus amino acid examined, although small increases were sometimes obtained in the others during later periods of incubation, e.g. 30–90 min. Phosphate and arsenate usually caused increases in rate of about the same order, but only in the system of strain '4' plus ornithine did this rate equal that with AMP-5′. The decarboxylation of histidine by strain 'F' was not accelerated by any of the three substances tested.

Controls showed that in the absence of added amino acid, no CO_2 liberation was caused by incubation of organisms with phosphate, arsenate or AMP-5′.

Effect of pH. Most bacterial amino acid decarboxylases show the highest activity under slightly acid conditions, namely, pH 4–5 (Gale, 1946).

Table 4 shows that the addition of 0·01 M phosphate, or arsenate, or AMP-5′, was without effect at the optimum pH (4·5–5·15). Marked increases in the rate of decarboxylation occurred at pH 6·0 and the maximum increase was found at pH about 7·0. When a cell-free extract was used under similar conditions, AMP-5′ also caused a small increase in activity (about 15 %), on the acid side (pH 4·22) of the pH optimum (5·10 in this experiment). With extracts, the largest increases in rate also occurred at pH 6·5–7·0.

Similar results were obtained with the six other strains of *Esch. coli* tested, and with the system *Cl. welchii*–glutamate–AMP-5′. An exception was the decarboxylation of ornithine by *Esch. coli* '8571', where the accelerating effect of AMP-5′ was greater at pH 4·48 than at pH 6·70 (+158 % compared with +21 %).

Effect of the concentrations of phosphate, arsenate and AMP-5′. At pH 6·7–6·8 the rate of decarboxylation of arginine by *Esch. coli* 'F' increased with increasing concentrations of phosphate or arsenate (Table 5) and AMP-5′ (Table 6) within the ranges tested. That these stimulations were not unspecific salt effects is shown by the facts that 0·25 M-NaCl inhibited the decarboxylation by about 15 % and 0·25 M-Na_2SO_4 by about 52 % (see also Gale & Epps, 1944). The maximum rate obtained with 0·7 M-phosphate at pH 6·8 was still lower (50 %) than that observed without phosphate at the pH optimum in acetate buffer (pH 4·5).

The time curve of the decarboxylation of amino acids is known to show in most cases a progressive decline in the rate (see, for example, Fig. 1) which cannot be explained by inhibition of the reaction by its products or by the fall of the substrate concentration. Gale (1940) showed that some decline was due to the thermolability of the enzyme. The activity never disappears completely, so that the reaction, given time, goes to completion.

The accelerating effects of phosphate, arsenate and AMP-5′ are usually most pronounced during the first 30 min. of incubation, before the enzyme

Table 3. *Effects of phosphate, arsenate and AMP-5′ on bacterial decarboxylation of* L-*amino acids*

Whole cells (2–10 mg. dry wt.); 5×10^{-4} M Cetavlon; 0·025 M citrate buffers, pH 6·62–6·80; 0·025 M L-amino acids; 0·01 M other additions at pH 6·7–6·8; final vol. 2 ml. Gas phase, N_2. Temp., 25°. Results are expressed as Q_{CO_2} for the first 30 min. incubation after addition of amino acid from the side arm.

Organism	Amino acid	Q_{CO_2} No addition	Phosphate	Arsenate	AMP-5′
Esch. coli 'F'	Arginine	42·1	76·4	74·6	107·0
	Ornithine	36·8	76·5	56·4	82·4
	Lysine	9·6	7·8	8·2	23·9
	Histidine	55·0	45·7	48·4	51·2
Esch. coli N.C.T.C. 86	Arginine	27·5	38·0	33·5	53·5
	Ornithine	16·5	25·0	19·5	31·5
Esch. coli '4'	Arginine	12·8	9·5	10·0	37·5
	Ornithine	52·0	101·5	88·0	95·0
	Lysine	27·3	42·9	55·2	97·0
Esch. coli N.C.I.B. 8571	Arginine	1·8	1·9	2·2	5·5
Esch. coli N.C.I.B. 8114	Arginine	18·8	17·8	19·6	46·8
Esch. coli 'M/48'	Arginine	16·5	11·4	12·5	36·0
Esch. coli 'T'	Arginine	7·5	7·1	8·4	13·5
Cl. welchii S.R. 12	Glutamate*	85·4	62·0	60·7	91·4

* This expt. at pH 6·42.

Table 4. *Effect of* pH *on the stimulation of the decarboxylation of arginine caused by phosphate, arsenate and AMP-5′*

Different whole-cell suspensions of *Esch. coli* 'F' were used in the three experiments: 3·7, 9·25 and 3·3 mg. dry wt. respectively in Expts. 1–3; 5×10^{-4} M Cetavlon; 0·025 M L-arginine; test substances all 0·01 M; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Buffer (M)	Final pH	Test substance	Total CO_2 liberated in 30 min. (μl.) No addition	With test substances	Increase (%)
1	Acetate–veronal (0·096)	4·75	Phosphate	1090	1162	+6·6
		5·15	Phosphate	1154	1113	0
		5·40	Phosphate	834	877	+5·2
		5·95	Phosphate	245	361	+47·4
		6·50	Phosphate	72	156	+116·8
		6·80	Phosphate	56	100	+78·6
		7·25	Phosphate	26	48	+84·6
2	Phthalate (0·05)	4·48	Arsenate	1164	1240	+6·5
	Phthalate (0·05)	5·50	Arsenate	610	695	+13·9
	Citrate (0·05)	6·62	Arsenate	117	244	+108·5
	Acetate–veronal (0·06)	7·05	Arsenate	32	62	+93·6
3	Phthalate (0·05)	4·48	AMP-5′	1087	1025	0
		4·95	AMP-5′	1034	992	0
		5·50	AMP-5′	721	765	+6·1
		5·82	AMP-5′	546	679	+24·4
	Citrate (0·05)	6·75	AMP-5′	264	423	+60·2
	Tris (0·05)	7·00	AMP-5′	138	289	+109·3
		7·40	AMP-5′	88	150	+70·5
		7·85	AMP-5′	55	64·5	+17·3

Table 5. *Effect of phosphate and arsenate concentration on the decarboxylation of* L-*arginine by* Esch. coli '*F*' *at* pH 6·7–6·8

A volume (0·25 ml.) of *Esch. coli* (7 mg. dry wt.) was stored for 7 days at −15°; 0·005 M citrate buffer, pH 6·7; 0·05 M L-arginine; sodium phosphate buffer, pH 6·8; sodium arsenate buffer, pH 6·7; final vol. 2·0 ml. Gas phase, N_2. Temp., 40°.

Phosphate (M)	Arsenate (M)	μl. of CO_2 liberated in 10 min.	30 min.	60 min.
0	0	142	195	224
0·01	0	198	306	350
0·05	0	305	555	653
0·25	0	410	931	1182
0	0·01	193	284	325
0	0·05	333	585	680
0	0·25	391	858	1072
0·01	0·01	208	360	421
0·05	0·05	362	732	888

Table 6. *Effect of AMP-5′ concentration on the decarboxylation of* L-*arginine by dialysed cell-free extracts of* Esch. coli '8114' *at* pH 6·7

Enzyme 8·4 mg. dry wt.; 0·025 M-citrate buffer, pH 6·70; 0·025 M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

AMP-5′ concn. (M)	Total CO_2 (μl.) liberated in 30 min.	60 min.	Increase in 60 min. (%)
0	44·5	62·5	—
0·0005	55·5	70·5	12·8
0·001	71·5	92	47·2
0·0025	86·5	109	74·5
0·005	107·5	127·5	104·0
0·01	147	171	173·8
0·02	250	299	378
0·04	349	462·5	640

activity has declined appreciably. After about 90 min. the rate in the presence of the accelerator often fell to that of the control vessel. Fig. 1 shows a representative rate curve where 0·005 M AMP-5′ was the accelerator.

The amounts of extra CO_2 evolved before the rates became equal were proportional to the amounts of phosphate, arsenate or AMP-5′ added. This ratio of moles of extra CO_2/mole of activator was approximately constant in any one experiment when the quantity of addition was varied. In the experiments recorded in Tables 7 and 8, ratios of 0·5:1 were found for arsenate and phosphate, and 1:1 for AMP-5′. The ratio was not altered by varying the enzyme concentration (Table 8). The same ratios were obtained with cell-free extracts, acetone-dried powder preparations and whole cells treated with Cetavlon, but not in every case. Occasionally lower ratios were obtained (i.e. less 'extra CO_2' was evolved/mole of accelerator added), particularly

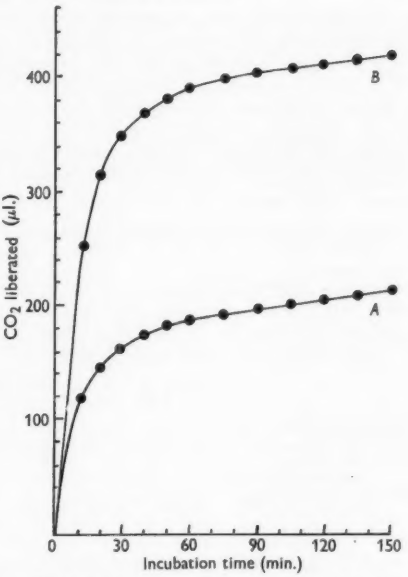

Fig. 1. Typical rate curves showing arginine decarboxylation with and without accelerator. *Esch. coli* '8114' acetone-dried powder (20 mg. dry wt.); 0·025 M-citrate buffer, pH 6·62; 0·025 M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Curve *A*, No additions; curve *B*, 0·005 M AMP-5′ added (= 224 μl.).

when fresh whole cells were used. After several days' storage, when the enzyme activity had fallen, ratios nearer to the above values were found. In other words, the accelerating effects were greater in acetone-dried powder preparations or in aged cells which showed rather feeble decarboxylase activity in the control vessels.

Paper chromatography of trichloroacetic acid extracts of the flask contents after incubation showed that AMP-5′ remained unchanged. No formation of adenosine, adenine, ribose 5′-phosphate, inorganic phosphate or ammonia was detectable, and when arginine was the amino acid decarboxylated only agmatine could be detected as end product apart from CO_2. No citrulline, ornithine or ammonia was formed.

Comparison of the effects of pyridoxal phosphate and AMP-5′. From the work of Baddiley & Gale (1945) and Umbreit & Gunsalus (1945), it is known that pyridoxal phosphate is the coenzyme of many bacterial amino acid decarboxylases, and it was of interest therefore to compare its effects with those of AMP-5′. Very small quantities of pyridoxal phosphate (e.g. 10 μg.) were sufficient to give considerable stimulation of decarboxylations; AMP-5′,

on the other hand, was required in much higher concentrations (e.g. 7 mg.) to give a similar increase (Table 9). The outstanding difference between the effects of the two substances was the pH dependence. Pyridoxal phosphate stimulated at the optimum pH of arginine decarboxylase (about pH 5), whereas AMP-5′ did not. At pH 6·7 and 7·0, on the other hand, the pyridoxal phosphate was much less effective than AMP-5′ (see Table 9). Similar observations were made with dialysed cell-free extracts of strain '8114' and with glutamic decarboxylase of *Cl. welchii* (whole cells). When both pyridoxal phosphate and AMP-5′ were added at this pH range the effects were slightly greater than the sum of the effects of the substances added alone.

Table 7. *Ratio of amounts of activator added and extra* CO_2 *liberated during arginine decarboxylation by* Esch. coli '*F*'

Whole cells of *Esch. coli* 'F' were stored for 19 days at 0° (3·7 mg. dry wt./vessel); 5×10^{-4}M Cetavlon; 0·05M citrate buffer, pH 6·77; 0·03M L-arginine; 0·005M and 0·01M phosphate, arsenate, and AMP-5′ at pH 6·7–6·8; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. At the end of 100 min., when the rate of CO_2 evolution was the same in all vessels, 0·5 ml. of 10% (v/v) H_2SO_4 was tipped in to release bound CO_2.

	None	Phosphate		Arsenate		AMP-5′	
Additions (μmoles)	0	10	20	10	20	10	20
Total CO_2 (μmoles)	9·0	14·5	18·7	14·5	19·1	18·8	28·0
Extra CO_2 (μmoles)	—	5·5	9·7	5·5	10·1	9·8	19·0
Ratio: $\frac{\mu\text{moles extra } CO_2}{\mu\text{mole additions}}$		0·55	0·49	0·55	0·51	0·98	0·95
		Av. 0·52		Av. 0·53		Av. 0·965	

Table 8. *Effect of enzyme concentration on the ratio of AMP-5′ added to extra* CO_2 *liberated*

Acetone–ether-dried powder of *Esch. coli* '8114'; 0·025M citrate buffer, pH 6·62; 0·025M L-arginine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Bound CO_2 was released by tipping acid when CO_2 evolution had declined to equal rates in each pair.

Enzyme added (mg.)	5		10		20	
AMP-5′ added (μmoles)	0	10	0	10	0	10
Incubation time before acid tip (min.)	150	150	105	105	90	90
Total CO_2 (μmoles)	5·2	14·9	6·5	15·3	8·8	18·1
Extra CO_2 (μmoles)		+9·7		+8·8		+9·3
Ratio: $\frac{\mu\text{moles extra } CO_2}{\mu\text{mole AMP-5′}}$		0·97		0·88		0·93

Table 9. *Comparison of the effects of AMP-5′ and pyridoxal phosphate on arginine decarboxylation by cell-free extracts and whole cells of* Esch. coli '*F*'

Buffer (0·025M); 0·025M L-arginine; 3 mg. dry wt. of cell-free extract in Expts. 1 and 3; 8·5 mg. dry wt. of whole cells $+5 \times 10^{-4}$M Cetavlon in Expt. 2; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Buffer	pH	Additions	Q_{CO_2} for 0–30 min. period
1	Phthalate	4·95	Nil	143·2
	Phthalate	4·95	AMP-5′ (0·01M)	142·6
	Phthalate	4·95	Pyridoxal phosphate (10 μg.)	321·0
	Phthalate	4·95	Pyridoxal phosphate (100 μg.)	350·0
	Phthalate	4·95	Pyridoxal phosphate (100 μg.) + AMP-5′ (0·01M)	356·5
	Citrate	6·70	Nil	22·0
	Citrate	6·70	AMP-5′ (0·01M)	54·5
	Citrate	6·70	Pyridoxal phosphate (100 μg.)	30·4
	Citrate	6·70	Pyridoxal phosphate (100 μg.) + AMP-5′ (0·01M)	71·0
	Tris	7·00	Nil	9·0
	Tris	7·00	AMP-5′ (0·01M)	51·3
	Tris	7·00	Pyridoxal phosphate (10 μg.)	19·3
	Tris	7·00	Pyridoxal phosphate (100 μg.)	22·0
	Tris	7·00	Pyridoxal phosphate (100 μg.) + AMP (0·01M)	70·4

Effects of other nucleotides. During the first 30 min. of incubation of arginine decarboxylase with substrate, yeast adenylic acid increased the rate of CO_2 evolution even more than an equal concentration of AMP-5′; the final yield of extra CO_2 was usually about the same, i.e. 1 mole of yeast adenylic acid or AMP-5′ caused the liberation of 1 mole of extra CO_2. As yeast adenylic acid is known to be a mixture of adenosine 2′-phosphate and adenosine 3′-phosphate these two nucleotides were examined separately, and together. Table 10 shows that with strain '86' the relative activations in the first 30 min. of incubation were: AMP-2′ > AMP-5′ > AMP-3′; AMP-2′ was about twice as effective as AMP-3′. AMP-2′ and AMP-3′, added together in equal amounts (0·005 M), gave the same result as 0·01 M yeast adenylic acid. Paper-chromatographic analysis according to Krebs & Hems (1953) and Carter (1950) showed that none of the nucleotides had changed during the incubations and that there was no enzymic conversion of AMP-3′ into AMP-2′. Other strains of *Esch. coli* ('F', '8114' and '4') and *Cl. welchii* gave results similar to those shown in Table 10.

As shown earlier (Table 1), ATP was much less effective than ADP or AMP, except in deep-frozen cells. Further experiments showed that the effect varied with the purity of ATP samples. Impure commercial preparations of ATP containing some unidentified organic phosphates were often more effective than AMP-5′.

Inosine triphosphate and inosine 5′-phosphate, both free from other phosphates, were found to be as effective as AMP-5′. Flavin mononucleotide also caused some activation but only about 50 % of that caused by AMP-5′. Guanylic acid did not increase the rate of arginine decarboxylation by strains 'F' and '4', and was slightly inhibitory. Uridylic acid and cytidylic acid had little or no effect under these conditions.

Effects of other substances on the rate of decarboxylation. Inorganic pyrophosphate caused some acceleration in the rate of arginine decarboxylation but was not as effective as an equal concentration of inorganic phosphate. Ribose 5-phosphate was as effective as AMP-5′; glucose 1-phosphate, glucose 6-phosphate and fructose 1:6-diphosphate gave small effects. Di*iso*propyl phosphorofluoridate (0·025 M) caused less stimulation than 0·025 M inorganic phosphate during the first hour of incubation with strain 'F' but more in the second and third hours. An effect was also found with 0·025 M diethyl *p*-nitrophenyl phosphate, but this was much smaller than the inorganic phosphate effect. Tetraethyl pyrophosphate (0·025 M) gave increases similar to those of inorganic phosphate during the first hour, but the effects gradually increased so that at the end of 3 hr. the extra CO_2 obtained was more than four times that obtained with inorganic phosphate. When compared with AMP-5′, tetraethyl pyrophosphate (0·005–0·02 M) was about three times as active during arginine decarboxylation by strain 'F', and about twice as active as AMP-5′ during lysine decarboxylation by strain '4'. Even after incubation for 6 hr. with tetraethyl pyrophosphate, extra CO_2 was still being liberated, though at a reduced rate. At pH 5·2, however, 0·005 M-tetraethyl pyrophosphate completely inhibited the decarboxylation of lysine and 0·01 M inhibited the decarboxylation of arginine (Table 11).

Four organic arsenicals which caused stimulations in strain 'F' were cacodylate, phenylarsonic acid, *o*-aminophenylarsonic acid, and arsonoacetate. Their activities, compared with that given by inorganic arsenate, were 127, 74, 54 and 112 % respectively.

Substances which had no effect on the decarboxylation of arginine by strain 'F' included adenosine, adenine, inosine, glucose, carbamyl phosphate,

Table 10. *Comparative effects of the adenosine monophosphates on the decarboxylation of arginine*

Expt. 1: 10 mg. of acetone–ether-dried powder of *Esch. coli* '8114'; 0·025 M-citrate buffer, pH 6·62; 0·025 M arginine. Expts. 2 and 3: 9·45 mg. (dry wt.) of whole cells of *Esch. coli* '86'; 5×10^{-4} M Cetavlon; 0·025 M phosphate buffer, pH 6·80; 0·0125 M arginine. Final vol. in all, 2 ml. Gas phase, N_2. Temp., 25°.

Expt. no.	Additions	CO_2 liberated (μl./30 min.)	Extra CO_2 (μl.)
1	Nil	71	—
	AMP-5′ (0·005 M)	187	116
	Yeast adenylic acid (0·005 M)	221	150
2	Nil	191	—
	AMP-2′ (0·01 M)	356	165
	AMP-3′ (0·01 M)	276	85
	AMP-5′ (0·01 M)	314	123
3	Nil	233	—
	AMP-2′ (0·005 M)	333·5	100·5
	AMP-3′ (0·005 M)	282·5	49·5
	AMP-2′ (0·005 M) + AMP-3′ (0·005 M)	398	165
	Yeast adenylic acid (0·01 M)	398·5	165·5

ethylenediaminetetraacetic acid, K^+, Mg^{2+} and Mn^{2+} ions, and nucleic acid from thymus gland (British Drug Houses Ltd.). Suspensions of *Esch. coli* 'F' (20 mg. dry wt.) which had been heated for 10 min. at 100° caused some inhibition. Of other phosphates tested, dimethyl nitrophenyl phosphorothioate and dimethyl dichlorovinyl phosphate were without effect. At 0·025M concentrations octamethylpyrophosphoramide, phenyl phosphate, thiamine phosphate, thiamine pyrophosphate and α-glycerophosphate were all inhibitory. Agmatine (0·005–0·02M), one of the end products of arginine decarboxylation, had no effect on the rate of CO_2 evolution.

Plant and animal tissue decarboxylases

Three plant tissues were examined for L-glutamic acid decarboxylase activity in the filtered juice (see Methods) at pH 6·8: carrot root and the fruits of water melon and vegetable marrow. Marrow juice was about twice as active as carrot juice, and about four times as active as water-melon juice. Most of the experiments were therefore carried out with marrow juice.

When filtered marrow juice was incubated with glutamate at pH 6·8 and 25°, the addition of phosphate, arsenate or AMP-5′ had relatively small effects on the CO_2 evolution, but major effects were found with dialysed enzyme, especially with dialysed extracts of acetone-dried powder preparations. In Table 12 experiments with the latter type of enzyme preparation are shown, carried out at pH 5·50 (the optimum) and pH 6·62. At the higher pH, phosphate (0·01M) and arsenate (0·01M) each caused a threefold increase in the rate of decarboxylation, and AMP-5′ (0·01M) and yeast adenylic acid (0·01M) caused about an eightfold stimulation. At this pH, pyridoxal phosphate only doubled the rate of CO_2 evolution. On the other hand, at the optimum pH (5·5), pyridoxal phosphate was the only substance of the five compounds tested which caused a substantial stimulation. As with the bacterial enzymes, the plant enzyme was not activated by uridylic or cytidylic acids, and guanylic acid was slightly inhibitory. Marrow glutamic decarboxylase thus behaves similarly to the bacterial enzymes.

The decarboxylating activities of animal tissues are known to be much weaker than those of either bacteria or plants (see Schales, 1951). The glutamic decarboxylase of brain, and the liver enzymes decarboxylating cysteic acid and 3:4-dihydroxy-L-phenylalanine were examined at pH 6·8. Increased rates were found on addition of phosphate, arsenate, pyridoxal phosphate plus phosphate and pyrophosphate. Pyridoxal phosphate alone had almost no effect. Similar observations have already been made by other workers, e.g. Bergeret, Chatagner & Fromageot (1955); Hartman, Akawie & Clark (1955). Yeast and muscle adenylic acids caused slight inhibitions, and other substances, which stimulated the bacterial decarboxylases but were found to have no effect on the animal-tissue decarboxylases, included cacodylate, tetraethyl pyrophosphate and di*iso*propyl phosphorofluoridate.

Table 11. *Comparison of the effects of tetraethyl pyrophosphate (TEPP) and AMP-5′ on the decarboxylations of arginine and lysine at* pH 6·8 *and* 5·2

Esch. coli 'F' (11·9 mg. dry wt.) was used for arginine decarboxylation; *Esch. coli* '4' (8·3 mg. dry wt.) was used for lysine decarboxylation; 5×10^{-4}M Cetavlon; 0·0625M tris–maleate buffers; 0·05M L-arginine and L-lysine; final vol. 2·0 ml. Gas phase, N_2. Temp., 25°.

pH	Amino acid	Incubation time (min.)	CO_2 liberated (μl.) No addition	0·01M AMP-5′	0·005M TEPP	0·01M TEPP	0·02M TEPP
6·8	Arginine	60	321	445	—	700	1047
5·2	Arginine	60	1081	1105	1276	6	—
6·8	Lysine	60	363	537	556	616	—
6·8	Lysine	360	954	1349	1377	1771	—
5·2	Lysine	60	309	327	28	14	—

Table 12. *Effects of phosphate, arsenate, adenosine monophosphates and pyridoxal phosphate on glutamate decarboxylation by dialysed extracts of marrow*

Acetone-dried powder of marrow (4·8 mg.) dialysed for 30 hr. at 0°; 0·025M-buffers; 0·025M L-glutamate; 0·01M additions except pyridoxal phosphate (20 μg.); final vol. 2·0 ml. Gas phase, N_2. Temp., 25°. Data refer to Q_{CO_2} for 0–30 min.

Buffer	Additions None	Phosphate	Arsenate	AMP-5′	Yeast adenylic acid	Pyridoxal phosphate
Citrate, pH 6·62	5·2	16·7	16·7	39·0	42·5	12·5
Phthalate, pH 5·50	28·2	32·7	30·2	28·6	28·2	43·4

Thus amino acid decarboxylases of animal tissues differ in some respects from those of bacteria and plants.

DISCUSSION

The experiments show that the activity of various amino acid decarboxylases of bacteria and plants can be increased by several inorganic and organic phosphates and arsenates. AMP-5′, inosine 5′-phosphate, AMP-2′ and tetraethyl pyrophosphate are particularly active. Activation takes place only near neutrality (around pH 6·8), and not at the pH optimum of the enzymes (around pH 4·5). In contrast, pyridoxal phosphate, the natural coenzyme, shows maximum stimulation at the optimum pH. The amino acid decarboxylases of animal tissue which have pH optima near neutrality are activated by pyridoxal phosphate but not by the organic phosphates and arsenates which are effective in bacteria and plants.

The quantities of the various compounds required for stimulation are of the order of mg./ml., whereas pyridoxal phosphate at the pH optimum acts in amounts of μg./ml. Although adenosine phosphates and inosine 5′-phosphate, as well as ribose 5-phosphate, all activate the bacterial decarboxylases, guanosine phosphate and pyrimidine nucleotides are inactive (only the mixed 2′- and 3′-phosphates were tested; the 5′-phosphates of these nucleosides were not available).

The quantitative nature of the activation of amino acid decarboxylations by AMP-5′ is comparable with other enzymes recently found to be activated by this nucleotide. For example, Tager & Rautanen (1955) found that the degree of stimulation by AMP-5′ of sulphite oxidation in oat mitochondria was a function of the AMP-5′ concentration. Madsen & Cori (1955) found that muscle phosphorylase *a* binds 4 moles of AMP-5′/mole of protein and phosphorylase *b* binds 2 moles of AMP-5′/mole of protein, and that whereas the *b* enzyme has an absolute requirement for AMP-5′, the *a* enzyme is still 65 % active in its absence.

Other enzymes requiring AMP-5′ for activity include the diphosphopyridine nucleotide-activated *iso*citric dehydrogenase of yeast (Kornberg, 1955) and of *Aspergillus niger* (Ramakrishnan & Martin, 1955). The fact that aged enzyme preparations of *Esch. coli* were more rapidly stimulated by AMP-5′ than were fresh enzymes has been reported in the present paper, and it is interesting to note that Sutherland (1955) has found that AMP-5′ and inosine 5′-phosphate increase the activity of liver polysaccharide phosphorylases to a greater extent in aged preparations than in fresh ones.

The stimulation of bacterial arginine and lysine decarboxylases by the cholinesterase inhibitor tetraethyl pyrophosphate is rather surprising, particularly as the degree of stimulation is much higher than those obtained with inorganic orthophosphate or pyrophosphate or AMP-5′. The fact that concentrations of TEPP which cause large activations at around neutral pH also inhibit the decarboxylations completely at optimum pH is of considerable interest and is being investigated further.

The results presented in this paper lead to the suggestion that there is probably a phosphate-containing substance which acts as a metabolic regulator, in that it can control the activity of the amino acid decarboxylases in the living cell. This metabolic regulator is more likely to be an organic rather than an inorganic compound, but its exact nature is open to speculation.

SUMMARY

1. The addition of cetyltrimethylammonium bromide to whole cells of *Escherichia coli* greatly increased the rate of amino acid decarboxylation. The detergent had no effect on extracts. The effect in whole cells is taken to be due to increased permeability.

2. Inorganic phosphate, arsenate and organic phosphates, especially adenosine phosphates, have been found to increase the rate of several bacterial amino acid decarboxylases when the pH is near the neutral point, but not at the optimum pH of the enzymes (pH 4–5).

3. The increases in decarboxylation rate were found to be proportional, within the range tested, to the amounts of phosphate, arsenate, or adenosine 5′-phosphate (AMP-5′) added. Two moles of phosphate or arsenate, or 1 mole of AMP-5′, caused the decarboxylation of up to 1 extra mole of amino acid. The maximum increases were obtained with aged enzyme preparations.

4. In contrast to the increases in rate obtained with AMP-5′, pyridoxal phosphate stimulated only at the optimum pH of the bacterial enzymes, and had little or no effect near the neutral point.

5. The relative activating powers of the adenosine monophosphates during the early stages of arginine decarboxylations were: adenosine 2′-phosphate > AMP-5′ > adenosine 3′-phosphate, adenosine 2′-phosphate being about twice as effective as adenosine 3′-phosphate. The total CO_2 liberated, however, was about the same. All three nucleotides were found to remain unchanged.

6. Adenosine diphosphate, inosine 5′-mono- and tri-phosphates, flavin mononucleotide, ribose 5-phosphate, and some other organic phosphates, especially tetraethyl pyrophosphate, all increased the rate of arginine decarboxylation. Similar effects were obtained with four organic arsenicals: cacodylate, phenylarsonic, *o*-aminophenylarsonic and

arsonoacetic acids. Guanylic, uridylic and cytidylic acids were among many compounds found to have little or no effect.

7. Plant tissue L-glutamic acid decarboxylases (*Cucurbita*, *Daucus carota*) were found to behave like the bacterial enzymes with regard to their stimulation by phosphate, arsenate, nucleotides and pyridoxal phosphate.

8. Under similar conditions the activity of animal tissue decarboxylases (L-glutamic, cysteine, 3:4-dihydroxy-L-phenylalanine) were not increased by adenine nucleotides, other phosphates or arsenates.

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A Crystallographic Study of some Derivatives of Gramicidin S

By G. M. J. SCHMIDT, DOROTHY CROWFOOT HODGKIN and BERYL M. OUGHTON
Chemical Crystallography Laboratory, University Museum, University of Oxford

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We first began the investigation of gramicidin S in 1944 at the suggestion of Dr R. L. M. Synge, to whom we are indebted for all the material used in the research. It appeared to us to be particularly interesting as a naturally occurring peptide of relatively low molecular weight, the study of which might throw light on the arrangement of amino acid residues in proteins. Further, it crystallized with surprising ease in crystals large enough for X-ray single-crystal analysis and it contains a functional group, the δ-amino group of ornithine, which made it possible to prepare a number of derivatives of the peptide, some containing heavy atoms. The present paper gives a survey of X-ray measurements on several of these gramicidin S derivatives, undertaken as a preliminary to an attempt at a detailed X-ray analysis.

Gramicidin S was originally isolated in the form of a hydrochloride by Gause & Brazhnikova (1944). It has been shown by Synge and others (Consden, Gordon, Martin & Synge, 1947; Synge, 1948) to be a cyclic peptide built up from five different amino acid residues occurring in unimolecular proportions. These residues are L-valine, L-ornithine, L-leucine, D-phenylalanine and L-proline, written in the sequence in which they are combined in the molecule as indicated by the examination of partial hydrolysates.

When the peptide was first examined crystallographically, neither its composition nor its molecular weight was known, and our first aim was to derive from the X-ray measurements the molecular complexity of the peptide. This proved unexpectedly difficult. The peptide derivatives examined crystallize from ethanol or acetone which is present as solvent of crystallization in crystals immersed in their mother liquor, and a variable amount of this solvent is lost on exposure to the air. For this reason the mass of material present in the crystal asymmetric unit is never an exact multiple of the minimum pentapeptide unit + solvent. From the formal relations between the different crystal structures studied it is clear that the molecule itself can be either a pentapeptide or a decapeptide. The latter alternative appears to be established by the synthesis reported by Schwyzer & Sieber (1956); it is also supported by diffusion and cryoscopic measurements (Pedersen & Synge, 1948; Belozersky & Paskhina, 1945), by the countercurrent distribution of its 2:4-dinitrophenyl derivatives (Battersby & Craig, 1951), and by the apparent close relation between gramicidin S and tyrocidine A, which has been shown by Battersby & Craig (1952) to have the same 5 amino acid sequence as gramicidin S, together with five other amino acids.

The fact that gramicidin S crystallizes from volatile solvents present in small bulk made it difficult in some cases to prepare specimens for X-ray photography in the usual way by drawing crystals in their mother liquor into capillary tubes, since the solvent evaporated too rapidly. Suitable crystals were therefore generally grown by slow cooling from warm saturated solutions sealed in fine-walled Hysil glass capillaries and photographed *in situ*, surrounded by their mother liquor. Large single crystals, several millimetres long, were often obtained by this technique.

RESULTS AND DISCUSSION

Table 1 summarizes the preliminary X-ray data collected on twenty different crystalline forms shown by nine different derivatives of gramicidin S. These substances are (*a*) the hydrochloride, sulphate, hydriodide, flavianate, rufianate, and chloroaurate; and (*b*) the *N*-acetyl, *N*-chloroacetyl, *N*-iodoacetyl derivatives, a molecular compound of the *N*-iodoacetyl derivative with iodine, and the *N*-(2:4-dinitrophenyl) derivative, prepared by Sanger (1946).

An appendix by Dr R. L. M. Synge provides chemical data on the different preparations

Table 1. *Data obtained by X-ray photography of single crystals of gramicidin S derivatives*

Derivative		*a*	*b*	*c*	*β*	Space group	*n**	*p*†
Hydrochloride (1)	Dry	24·4	34·8	18·9	—	$C222_1$	8	2
(2)	Dry	24·9	34·2	37·7	—	$C222_1$	8	4
	Wet	26·1	35·3	37·9	—	$C222_1$	8	4
	Dry	24·1	34·6	18·9	—	C222	8	2
Sulphate	Wet	25·8	34·1	37·8	—	$C222_1$	8	4
	Dry	24·4	33·5	18·9	—	C222	8	2
Hydriodide (1)	Wet	28·0	41·1	39·2	—	I222 or $I2_12_12_1$	8	4
	Dry	22·3	41·8	39·6	—	I222 or $I2_12_12_1$	8	4
(2)	Wet	28·0	41·0	39·0	—	I222 or $I2_12_12_1$	8	4
	Dry	23·2	41·2	38·8	—	$P2_12_12_1$	4	8
(3)	Dry	23·0	72·8	19·8	—	$P2_12_12_1$	4	8
Flavianate	Dry	48·5	36·6	18·8	—	$B22_12$	8	—
Rufianate	Wet	43	33	57	104°	C2	4	—
N-Acetyl	Wet	28·3	—	54·9	—	$P6_122$	12	3
	Dry	27·3	—	55·2	—	$P6_122$	12	3
N-Chloroacetyl	Wet	28·1	—	54·8	—	$P6_122$	12	3
	Dry	27·5	—	54·8	—	$P6_122$	12	3
N-Iodoacetyl	Wet	28·2	—	55·2	—	$P6_122$	12	3
	Dry	27·7	—	54·7	—	$P6_122$	12	3
N-Iodoacetyl + I_2	Dry	24·4	40·5	38·5	—	I222 or $I2_12_12_1$	8	4
Chloroaurate	Dry	21·1	38·5	40·5	—	$P22_12$	4	8
	Dry	21·1	38·5	40·5	—	I222 or $I2_12_12_1$	8	4
N-2:4-Dinitrophenyl	—	31	34	19	—	—	—	—

* *n*, number of asymmetric units in the unit cell.
† *p*, number of pentapeptide units in the crystallographic asymmetric unit.
a, *b* and *c* are in Å.

examined. They dated from three different periods—1943, 1945–47 and 1952. While the earliest materials showed small differences in unit cell dimensions from those here recorded, probably owing to the presence of peptide mixtures, a sample of the iodoacetyl derivative prepared from highly purified gramicidin S, specimen IV, examined in 1952, gave X-ray photographs identical with those obtained in 1945–47, showing that the small proportion of other peptide materials still present in the preparations at this date have no observable effect on the X-ray data.

Characteristics of wet and dry crystals of gramicidin S derivatives

Crystals of all the derivatives of gramicidin S that we have examined appear markedly birefringent and transparent and frequently show sharp, well-defined faces when covered with their mother liquor. On removal from this liquor they may become opaque and cracked, but slow drying often leaves them with both shape and transparency very little changed. X-ray photographs show that in all cases there are changes in the unit cell dimensions and in the relative intensities of certain of the X-ray reflexions. The reflecting power of the crystals diminishes on drying to a degree which depends on the rate of drying. Very slowly dried crystals show almost as many X-ray reflexions as wet crystals; these usually extend to spacings of approximately 1·5 Å.

Three types of change are observed during crystal drying, which are rather different from the changes observed with protein crystals. The most marked alterations occur with the hydrochloride and sulphate, where a change in crystal symmetry results in the halving of one unit cell dimension. All reflexions with l odd on the wet crystal photographs disappear, leaving the remaining reflexions comparatively little altered in position and intensity. The loss of solvent appears therefore to be accompanied by an adjustment of the positions of the molecules at z and $z+\frac{1}{2}$ in the wet crystal which renders them effectively identical in the dry crystal. Very little crystal shrinkage occurs but the changes in the molecular positions are not regular. Certain of the layer lines are markedly 'smeared' and diffuse reflexions appear, particularly from the hydrochloride. These phenomena are illustrated in Pl. 2*a* and *b*.

In the second type of change, that of the hydriodides, the unit cells of wet and air-dry crystals remain of the same order of magnitude, but there is a marked shrinking in one direction, that of the a axis, of approximately 5 Å. This change suggests the existence of a single layer of solvent molecules which is removed on drying; it is similar to changes that occur on drying protein crystals, but is accompanied again, in one of the gramicidin S hydriodide crystals, by a change of crystal symmetry.

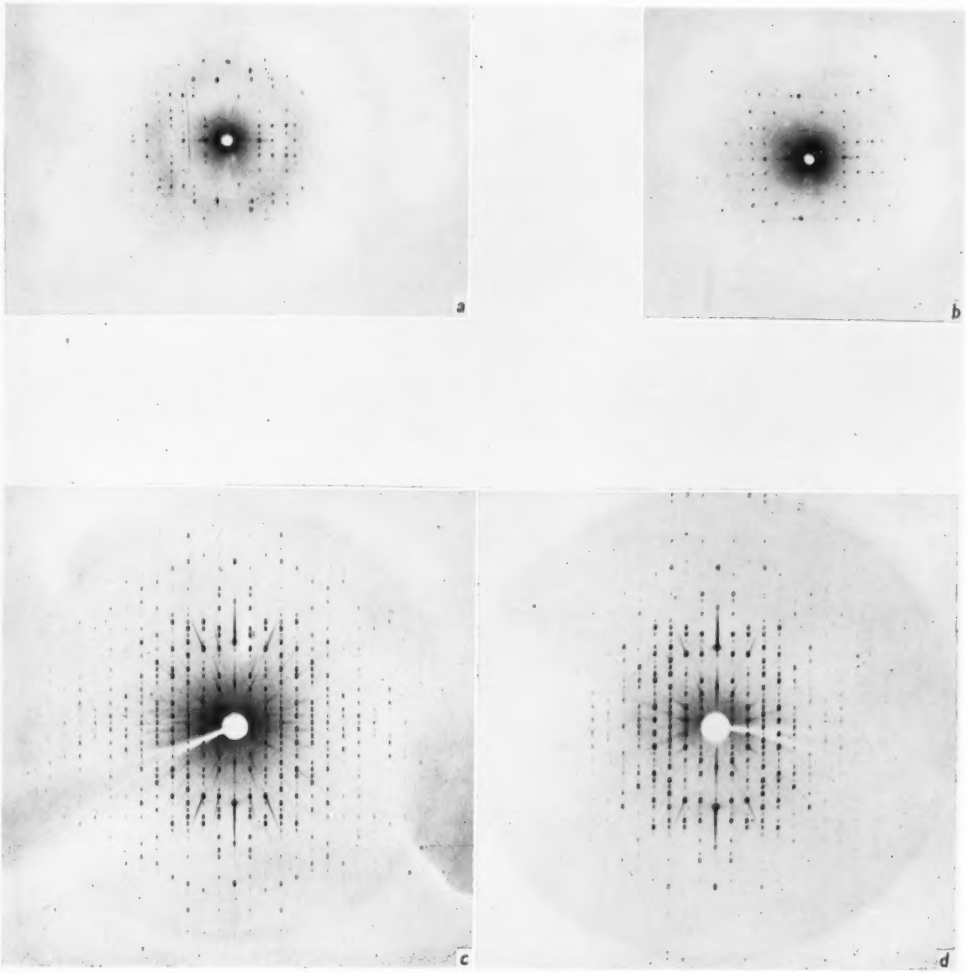
In the third type of change, that of the acetyl derivatives, there is very little alteration in the unit cell dimensions. There are, however, small changes in the intensities of some of the X-ray reflexions which indicate that here the solvent molecules are zeolitic, able to pass out of the lattice, leaving the position and orientation of the peptide units practically unchanged. The order of magnitude of the solvent loss on drying is illustrated by measurements by Dr Synge: he observed that a preparation of wet crystals of acetylgramicidin S from which all visible drops of water had been removed weighed 55 mg. After being dried in a vacuum desiccator at room temperature, it weighed 40·5 mg.; this weight increased to 43·3 mg. on exposure to the air.

Molecular weight of gramicidin S

The stoicheiometric unit. The determination of the minimum stoicheiometric molecular weight from the X-ray data depends on the accuracy with which the cell dimensions, density and solvent content of the crystals can be measured. So far no attempt has been made to measure the solvent content of the wet crystals, and the estimations are therefore derived from the air-dry crystals.

There are experimental difficulties in measuring both the cell dimensions and the density with high accuracy. The number of observed reflexions is smaller for some of the air-dry crystals than for the wet crystals, and these are often not sharp, owing to the disordered character of the dry crystals. The density measured by flotation in aqueous salt solutions is found to vary with the time of immersion of the crystals, presumably owing to solution entering the crystal lattice. For each crystal studied, the value for the density recorded in Table 2 is that observed for freshly wetted crystals and should certainly be regarded as a maximum; some solvent molecules may enter the crystals directly they are immersed. But it is probable that the main difficulty in obtaining a measure of the peptide molecular weight from X-ray data lies in the accurate determination of the solvent content of the air-dried crystals. This has usually depended on a measurement of solvent loss on drying *in vacuo* at 100°, followed by analysis of the dried crystals.

With gramicidin S crystals we observed a serious discrepancy at the beginning of our research with the hydrochloride, which is recorded in Table 2. The figure for the weight of the crystal unit less solvent lost on drying at 100° is very much higher than the chemically determined weight of a pentapeptide unit, even if this is associated with one molecule of water of crystallization as suggested by the analytical data on the vacuum-dried crystal (Table 2). It seems clear that the air-dried crystals contain



(*a*) Oscillation photograph about the *c*-axis of wet gramicidin S sulphate. (*b*) Oscillation photograph about the *c*-axis of dry gramicidin S sulphate. (*c*) Precession photograph of dry *N*-chloroacetylgramicidin S, showing (0*kl*) reflexions. (*d*) Precession photograph of dry *N*-acetylgramicidin S, showing (0*kl*) reflexions.

G. M. J. SCHMIDT, DOROTHY CROWFOOT HODGKIN AND BERYL M. OUGHTON—
A CRYSTALLOGRAPHIC STUDY OF SOME DERIVATIVES OF GRAMICIDIN S

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Table 2*a*. *Molecular-weight data on gramicidin S hydrochloride*

Suggested formula of vacuum-dried crystals	Loss of wt. on drying at 100°	Mol.wt. of suggested formula	Minimum immersion density	X-ray mol.wt.
$C_{30}H_{46}O_5N_6,HCl,H_2O$	6·1%	624	1·217	687 × 2

Table 2*b*. *Molecular-weight data on N-acetylgramicidin S and its substitution products*

Derivative	Suggested formula of air-dried crystals	Mol.wt. of suggested formula	Minimum immersion density	X-ray mol.wt.
N-Acetylgramicidin S	$C_{32}H_{48}O_6N_6,C_2H_5.OH,1{\cdot}5H_2O$	685	1·165	687 × 3
N-Chloroacetyl-gramicidin S	$C_{32}H_{47}O_6N_6Cl,C_2H_5.OH,1{\cdot}5H_2O$	719·5	1·238	742 × 3
N-Iodoacetyl-gramicidin S	$C_{32}H_{47}O_6N_6Cl(0{\cdot}2)\,I(0{\cdot}8),C_2H_5.OH,1{\cdot}5H_2O$	793	1·345	816 × 3

more solvent than the analytical data suggest. Some attempt was made to check this idea by having the chemical analyses for chloroacetyl- and iodoacetyl-gramicidin S carried out on the air-dried instead of on vacuum-dried crystals. The analytical figures suggest that both ethanol and water are present in amounts which give much better agreement with the conclusions from X-ray data. Within the limits of error of both types of measurement it seems likely that the formula unit in the acetyl series of crystals approximate to 1 molecule of pentapeptide: 1 molecule of ethanol: 1·5 molecules of water, and this is shown in Table 2. But it should be realized that in crystals which are disordered to some extent the solvent molecules may well be present in fractional amounts as suggested in the analytical description in Appendix 1, and until a detailed X-ray analysis of the crystals can be carried out our knowledge of the contents of the crystals will remain rather imprecise.

Our attitude to our observations is naturally affected by our knowledge of the chemical constitution of gramicidin S. Our experience indicates that when X-ray methods are used to determine unknown molecular weights of this order of magnitude the errors most likely to occur may increase the estimated molecular weight by as much as 10%.

The absolute molecular weight. The smallest molecular-weight unit that appears as the asymmetric unit (the simplest repeating unit) in any of the crystal structures examined is the unit corresponding to a decapeptide, which is observed in the air-dry forms of gramicidin S sulphate and hydrochloride. However, in the hexagonal acetylgramicidin S series, the asymmetric unit contains three pentapeptide units. The first, most natural, deduction from these two observations is that the molecule itself may be a pentapeptide, which owing to complexities of packing sometimes occurs in two positions not related by the space-group symmetry of the crystal, and sometimes in three. But there is an alternative formal possibility, which is most likely. In the hexagonal space group $P6_122$ the twofold axes of symmetry present can be symmetry elements of the molecules themselves. In this case, the crystal asymmetric unit may be essentially three half decapeptide molecules, or $1\frac{1}{2}$ molecules, provided that the decapeptide molecule itself has a twofold axis of symmetry. Formally, therefore, the crystallographic evidence requires the molecule to be either a pentapeptide or a decapeptide having a twofold axis of symmetry. The decapeptide structure alone is in accord with its chemical synthesis and with the measurements carried out by Pedersen & Synge (1948) and by Battersby & Craig (1951).

Size and shape of the molecules

While it is not possible to make precise deductions about the size and shape of a molecule of the complexity of gramicidin S from our present data, there are certain features of these data which are suggestive and worth further comment.

Throughout the series of crystal structures one unit cell dimension (usually *c*) is 18·8–19·8 Å, or some multiple of this figure. In this direction a very strong reflexion, (004) in the hydrochloride, and (000,12) in the acetyl series, shows that many atoms are concentrated in layers about 4·8 Å apart. Pl. 2*c*, *d* illustrates the overwhelming strength of the (000,12) reflexion in *N*-chloroacetyl- and *N*-acetyl-gramicidin S. The formal consequences of this are illustrated in Fig. 1*a* by the section at $x=0$ in the three-dimensional Patterson series for *N*-acetyl-gramicidin S. The strong peak (A) shows that there are many vectors parallel to *c* between atoms 4·8 Å apart. The marked streaking along lines parallel to *a* suggests that these atoms are in layers.

The remaining two unit-cell dimensions, *a* and *b*, vary in their ratio to one another in the different crystal structures, but a close relationship can again be traced between the orthorhombic and hexagonal

structures; the conditions limiting the molecular size can best be seen from the latter type of structures. In Fig. 1*b* is shown the section of the *N*-acetylgramicidin S Patterson series calculated at $z = 0$. This indicates that the whole crystal structure repeats approximately at the positions $\frac{1}{3}a_1$, $\frac{2}{3}a_2$ and $\frac{2}{3}a_1$, $\frac{1}{3}a_2$. The character of the vector distribution is best understood if the molecules are rather compact. They need not be cylindrical but could have an elliptical or an approximately rectangular cross-section; if so, extreme values for the two sides of the rectangle might be set at about 11 and 19 Å. A decapeptide molecule having these dimensions would extend about 9·5 Å, or $c/6$, in the c direction. Thus, within the molecule would be two layers of atoms approximately 4·8 Å apart.

Detailed X-ray analysis of gramicidin S

Of the different crystal structures so far examined those of *N*-acetyl-, *N*-chloroacetyl- and *N*-iodo-acetyl-gramicidin S appear most suitable for use in attempting a detailed X-ray analysis. The compounds are very nearly isomorphous and, as Pl. 2*c* and *d* shows, some of the X-ray reflexions from corresponding crystal planes show considerable changes in intensity, from which the positions of the heavy atoms may be found and the phases of the reflexions subsequently inferred. The molecular arrangement is very complicated, but nevertheless presents a far simpler X-ray analytical problem than do the simpler proteins. We are accordingly collecting three-dimensional X-ray data from these crystals and attempting to find a detailed solution of their structure.

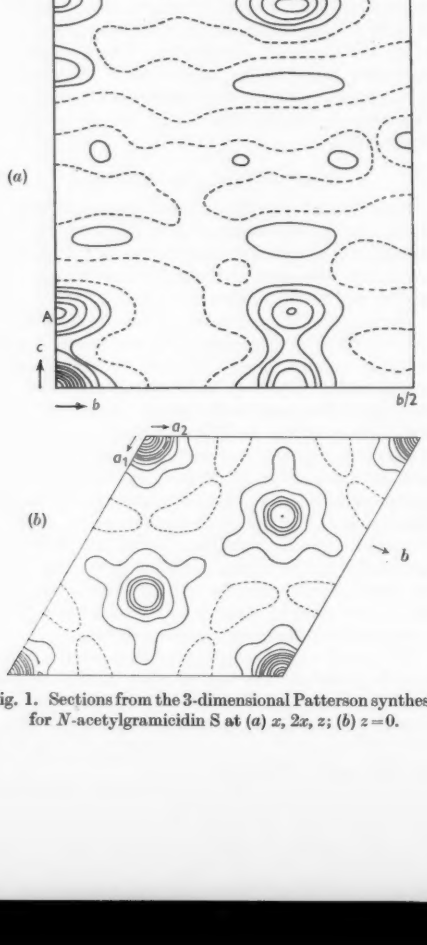

Fig. 1. Sections from the 3-dimensional Patterson synthesis for *N*-acetylgramicidin S at (*a*) x, $2x$, z; (*b*) $z = 0$.

CONCLUSION

More definite crystallographic knowledge of the character of the gramicidin S molecule must await the outcome of this detailed study. But already in this preliminary survey of evidence concerning the molecular weight, size and shape we have made certain observations which bear on the investigation of proteins. Our experiences in the molecular-weight determination of gramicidin S can, for example, be paralleled by measurements on insulin. It is noticeable that the best crystallographic value for the molecular weight of zinc insulin, 11 900, is numerically larger than the comparable figure, 11 554, derived by calculation from the amino acid composition; and this figure, 11 554, again is twice the actual minimum chemical stoicheiometric figure of 5777 (Sanger & Tuppy, 1951; Sanger & Thompson, 1953). This suggests that in insulin crystals, as in gramicidin S crystals, molecules may be fitted together in the crystal asymmetric unit in ways which are not governed by the symmetry operations of the crystal. Lastly our present evidence on the size, shape and symmetry of the gramicidin S molecule limits considerably the way in which the amino acid residues can be arranged within the molecule and has led us naturally to build a number of possible model structures. These structures appear to us to have interesting and conflicting relations with present theories of peptide-chain configurations in proteins. Since it may still be years before we can solve the detailed structure of gramicidin S we have described in Appendix 2 the models we have so far constructed and their relation to ideas of protein structure.

EXPERIMENTAL

The main practical problem in this work was the photography of crystals in the wet state. The method usually employed with crystalline proteins of drawing up the crystal into a capillary tube from its aqueous mother liquor proved impracticable, particularly so with the hydrochloride. Here slow evaporation of a concentrated solution in 75% (v/v) aqueous ethanol resulted in a mush of small

crystals; the solvent evaporated before a single crystal could be sorted out or introduced into the capillary. This difficulty was overcome by growing single crystals in thin-walled capillaries so that they could be photographed *in situ*. In this way large crystals were produced from 50 mm.^{3} of solution or less, i.e. recrystallization could be carried out on a micro scale.

The capillaries were blown from Hysil glass with an average diameter of 1 mm. and an average wall thickness of 20 μ. Hysil was chosen in preference to Lindemann glass because of the low pH of the solution. Hysil contains no Fe, and its constituents are of relatively low atomic weight. A suitable quantity of liquid (usually a column about 5 mm. long) with a small amount of solid was drawn up into the capillary which was then sealed at both ends in the micro-flame. The capillaries were immersed in a large, well insulated tank containing water whose temperature was controlled by an electric heating element with variable energy input. No precautions were taken to ensure a perfectly uniform temperature drop, but recrystallization was usually successful on cooling from about 75° to room temp. at an overall rate of 1° in 4 hr., and in favourable cases gave crystals up to 5 mm. long. The method has so far not been successful with the flavianate and the 2:4-dinitrophenyl derivative.

To compare wet with dry crystals our method at this stage was simply to break the capillary, drain the liquid by inserting a smaller capillary, and allow the crystal to dry slowly.

Copper radiation ($\lambda = 1{\cdot}542$ Å) was used for all the X-ray photography. Most photographs were taken on flat films with a crystal-to-plate distance of 4·0 cm.; with a pinhole slit system (second aperture 0·4 mm.) very clean photographs were obtained after exposures up to 48 hr. without special precautions. Cell dimensions were calculated either from layer line measurements on cylindrical films of 3 cm. radius, or from symmetrical photographs recording several orders of, say, ($h00$) and ($\bar{h}00$), taken for the most part on X-ray plates to avoid shrinkage errors. We cannot claim accuracy in cell dimensions greater than 2%. A few recent photographs were taken on a precession camera.

Gramicidin S hydrochloride

Gramicidin S HCl crystallizes very readily in small needles from aqueous ethanol; larger crystals were grown by slow cooling from 70% (v/v) aqueous ethanol. The crystals are elongated along [001], showing {110} and {001}.

$$\alpha \parallel a,\ \beta \parallel c,\ \gamma \parallel b.$$

The data given in Table 1 refer to two different batches, of which the first was less pure than the second. This first batch contained dry crystals of two kinds which gave different X-ray photographs, and are listed as (1) and (2). It is noticeable that the c-axis dimension of (2) is similar to that of the wet crystals of later preparations; probably they contained impurities which slowed down the normally rapid transformation of wet to dry crystal structures.

The stable dry forms of the two batches are very similar in cell dimensions, intensities of X-ray reflexions and the types of diffuse reflexions shown in X-ray photographs, i.e. particularly marked smear lines running through reflexions of index ($hk4$). One crystal photographed from the second batch showed unusual diffuse reflexions which indexed on a body-centred lattice with a doubled c axis.

Gramicidin S sulphate

The sulphate resembles the hydrochloride extremely closely. The crystals are needles, elongated along [001], showing {110} and {001}, also thin plates on {010}, showing {100} and {001}.

$$\alpha \parallel a,\ \beta \parallel c,\ \gamma \parallel b.$$

On drying, the crystals show disorder phenomena as with the hydrochloride. Marked smear lines appear on photographs through reflexions of index ($hk4$); on some photographs diffuse reflexions occur requiring a doubled and trebled a axis.

Gramicidin S hydriodide

Three forms were observed, of which one was prepared by Synge. The other two occurred in a preparation made at Oxford, which was slightly discoloured, suggesting the presence of some free iodine. Unfortunately no analytical data were obtainable.

Both modifications (1) and (2) were well-developed needles elongated along [001] and showing {110} and a variety of unidentified domal faces. Optically, both forms showed

$$\alpha \parallel a,\ \beta \parallel c,\ \gamma \parallel b$$

when wet; drying produced marked loss of birefringence, accompanied perhaps by a change in optical orientation.

Crystals of the third form are small, clear plates on {010}, found in one batch of Oxford crystals. They gave unusually good X-ray photographs, showing no sign of crystal disorder.

Gramicidin S flavianate

The flavianate has not been photographed in the wet state, since all attempts at recrystallization in tubes produced bunches of needles. The crystals are elongated along [001] and show {100} and {010};

$$\alpha \parallel c,\ \beta \parallel a,\ \gamma \parallel b.$$

The natural interpretation of the X-ray data on this crystal is that the unit cell contains sixteen decapeptide molecules and sixteen flavianic acid molecules. However, most preparations have shown, on analysis, higher contents of flavianic acid than this. Possibly some flavianic acid replaces gramicidin S at random in the lattice.

Gramicidin S rufianate

The rufianate was prepared at Oxford by mixing solutions of 2 mg. of gramicidin S HCl and 2 mg. of rufianic acid in absolute ethanol. The dark-red crystalline precipitate formed was filtered off, washed with 70% ethanol and recrystallized once from absolute ethanol. Large crystals, which were red needles, elongated along [010], were grown by slow cooling from 90% (v/v) aqueous ethanol.

On drying, the crystals lose their birefringence and become opaque; X-ray photography shows nearly complete breakdown of the crystal lattice.

N-Acetyl-, N-chloroacetyl- and N-iodoacetyl-gramicidin S

These three acyl derivatives are nearly isomorphous. They occur in beautifully-formed hexagonal needles showing {10$\bar{1}$0} and {10$\bar{1}$1}; occasionally also bipyramids with only {10$\bar{1}$1} are observed. They are optically negative. Very slow drying yields crystals with clear faces which give good measurable reflexions in the optical goniometer. In general

the pattern of the intensities of the X-ray reflexions is very similar in the three crystals, but some changes are observable both on drying and on halogen substitution. Analysis shows (see Appendix 1) that there is incomplete substitution of the Cl by I in the *N*-iodoacetyl derivative.

Iodoacetylgramicidin S crystallized in the presence of excess of iodine

An orthorhombic form of iodoacetylgramicidin S was obtained by Synge in 1952, by crystallizing it from aqueous ethanol in the presence of excess of iodine. Beautiful little brownish-red needle-shaped crystals were formed, elongated along [010] with well-developed {100} and other unidentified domal faces. These showed marked pleochroism, from which it could be deduced that the iodine molecules lay perpendicular to the needle axis. Unlike most of the derivatives examined, the needle axis is not the one which is a multiple of (18·8–19·8 Å), but the optics seem to bear the same relationship to the cell dimensions as in other crystals. $\gamma \parallel b$, and probably $\alpha \parallel a$ and $\beta \parallel c$.

Gramicidin S chloroaurate

The chloroaurate was prepared by Synge in 1952, and grows in the form of colourless, flat needles, elongated along [001], with well developed {100} or {010}.

Of several crystals selected, one was found to have crystallized in a different space group from the others. All of them had the same unit cell dimensions, and most seem to be body-centred, probably having the space group I 222; the one exception had the space group P22$_1$2.

The crystals were weakly birefringent, with $\alpha \parallel a$, $\beta \parallel c$ and $\gamma \parallel b$.

SUMMARY

1. Single crystals of a series of derivatives of gramicidin S have been prepared and examined by X-ray methods. Their morphology, unit cells and space groups have been determined, and a comparison between crystals in the wet and dry states has been made.

2. The weights of the crystal asymmetric units of the hydrochloride and *N*-acetyl, *N*-chloroacetyl and *N*-iodoacetyl derivatives have been determined by X-ray methods and the results compared with the molecular weights determined by chemical analysis.

3. It is concluded that the gramicidin S molecule is most probably a decapeptide in which two halves of the molecule are related by a twofold axis of symmetry. Some conclusions about the size and shape of the molecules are drawn.

4. Appendix 1 describes the methods of preparation and chemical analyses of the crystals examined.

5. In Appendix 2 possible molecular models for gramicidin S are discussed in relation to current theories of protein structure.

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APPENDIX 1

Preparation of some Derivatives of Gramicidin S

By R. L. M. SYNGE
*The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London S.W. 1, and The Rowett Research Institute, Bucksburn, Aberdeenshire**

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N (Kjeldahl) determinations were done by the incineration procedure of Campbell & Hanna (1937). Other elementary analyses, unless otherwise stated, were by Weiler and Strauss, Oxford. Evaporations, unless otherwise stated, were done *in vacuo* below 40°.

* Present address.

Starting material: gramicidin S hydrochloride. Specimens I and III (Synge, 1945; Consden, Gordon, Martin & Synge, 1947) were first sent for crystallographic study and used for preparing derivatives. Later preparations have all been crystallized from the same batch of crude material as specimen III. One such preparation was studied by countercurrent distribution by Dr Lyman C. Craig and colleagues

and gave 90–93% of the preparation in the main peak (see Craig, Gregory & Barry, 1950; Fig. 5). Dr Craig kindly returned the main-peak fraction (tubes 19–31), and this highly purified product is here described as specimen IV.

Gramicidin S sulphate. The hydrochloride (60 mg.) was dissolved in 70% (v/v) aqueous ethanol, and Ag_2SO_4, equivalent to the chloride present, was added in the same solvent. The resulting precipitate was centrifuged off. The combined supernatant and washings, on slow evaporation at atmospheric pressure, yielded the crystals which were sent for study.

Gramicidin S hydriodide. The sulphate was prepared as above but was not crystallized. The solution was treated with BaI_2 (also dissolved in 70% v/v aqueous ethanol) equivalent to the sulphate present. The resulting precipitate was centrifuged off, and the supernatant on slow evaporation at atmospheric pressure yielded large crystals which were sent for study. A more recent *preparation* (analysed air-dry by Pascher, Bonn) gave: C, 49·9; H, 7·1; N (Dumas), 10·5; I, 15·3%. $C_{60}H_{92}O_{10}N_{12},2HI,4H_2O,3C_2H_5.OH$ requires C, 49·4; H, 7·5; N, 10·5; I, 15·8%.

Gramicidin S flavianate. Gramicidin S hydrochloride (30 mg.) was dissolved in 1 ml. of 70% (v/v) aqueous ethanol and treated with 45 mg. of flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid) dissolved in 1 ml. of the same solvent. A crystalline precipitate formed immediately, and was filtered off, washed with the same solvent and recrystallized from warm aqueous ethanol. Yield, 35–45 mg. Different preparations have given very variable elementary analyses in the range 1–2 molecules of flavianic acid/decapeptide unit.

Gramicidin S chloroaurate. Gramicidin S hydrochloride (24 mg.) was dissolved in 70% (v/v) aqueous ethanol (2 ml.) and commercial 'brown gold chloride' (75 mg.) dissolved in the same solvent was added. Water was then added drop by drop to the resulting clear solution until turbidity resulted. The solution was then allowed to evaporate slowly, giving long yellow *needles* in bundles, from which the mother liquor was decanted. The needles were washed successively with 30% ethanol and water, and analysed air-dry. [Found: C, 41·9; H, 5·9; N (Kjeldahl), 9·0; Au (combustion residue), 19·3%.

$$C_{60}H_{92}O_{10}N_{12},1{\cdot}8HAuCl_4,0{\cdot}2HCl,2C_2H_5.OH$$

requires C, 41·4; H, 5·7; N, 9·1; Au, 19·2%.]

N-Acetylgramicidin S. Gramicidin S hydrochloride (40 mg.) was dissolved in pyridine (4 ml.) and acetic anhydride (1 ml.). After keeping for 24 hr. at room temp. the mixture was evaporated to dryness, and water was added to the resulting gummy product, in which it was insoluble. After repeated evaporation with additions of fresh portions of water, the residue was dissolved in 0·8 ml. of ethanol, water was added to turbidity, and evaporation was allowed to proceed slowly in air at room temp. Large crystals resulted, which were recrystallized in the same way. The behaviour of the solvent of crystallization is described in the main paper. Yield, after drying *in vacuo* at room temp. over H_2SO_4–soda lime, 40·5 mg. The *product* lost 4% of its wt. on further drying over P_2O_5 *in vacuo* at 100° and, after drying in this manner for analysis, had $[\alpha]_D^{22} - 313°$ in 70% (v/v) aqueous ethanol (*l*, 0·5; *c*, 1·5). [Found: C, 62·2; H, 7·7; N (Kjeldahl), 13·5%. $C_{64}H_{96}O_{12}N_{12},0{\cdot}5H_2O$ requires C, 62·2; H, 7·9; N, 13·6%.] No chloride was detected in the product by testing with $AgNO_3$ in aqueous ethanol, and amino N was absent (Van Slyke nitrous acid procedure, 30 min. reaction time).

N-Chloroacetylgramicidin S. Gramicidin S hydrochloride (100 mg.) was treated with water (20 ml.) and chloroform (B.P., 10 ml.). Na_2CO_3 (700 mg.) was added to the resulting mixture which was kept at 0°. Two portions of chloroacetyl chloride (each 0·13 ml.) were added with shaking at intervals of 5 min. and the mixture was shaken for a further 10 min. The chloroform layer was separated, and the aqueous layer extracted with two further 10 ml. portions of chloroform. On evaporation, the combined chloroform extracts yielded 90 mg. of clear brown gum, which was dissolved in 3 ml. of ethanol. Water was then added until crystallization started. This continued on allowing slow evaporation in air. The resulting large crystals were freed from mother liquor and washed by decantation successively with 30 and 10% (v/v) aqueous ethanol and then with several changes of water. The *product* (35 mg.) was analysed air-dry. [Found: C, 56·8; H, 7·5; N (Kjeldahl), 11·9; Cl (Carius), 5·3%.

$$C_{64}H_{94}O_{12}N_{12}Cl_2,3H_2O,1{\cdot}5C_2H_5.OH$$

requires C, 56·8; H, 7·7; N, 11·9; Cl, 5·0%.] No chloride was detected in the product with $AgNO_3$ in aqueous ethanol.

N-Iodoacetylgramicidin S. Crude *N*-chloroacetylgramicidin S (55 mg.) was dissolved in acetone (2 ml.) and NaI (90 mg.) dissolved in acetone (3 ml.) was added (cf. Conant, Kirner & Hussey, 1925). Immediate precipitation occurred, presumably of NaCl. The mixture was kept overnight. Water was then added and the precipitate dissolved; on addition of excess of water the derivative of gramicidin S was precipitated. The precipitate was washed thoroughly with water and dried *in vacuo* at room temp. over H_2SO_4–soda lime (yield, 56 mg.). The *product*, which was considerably less soluble in aqueous ethanol than *N*-chloroacetylgramicidin S, gave large crystals on slow evaporation in air at room temp. from solution in a mixture of water, ethanol and acetone. These were analysed air-dry. The iodine determination was kindly done by Mrs R. V. Pitt-Rivers of the National Institute for Medical Research. Chlorine was determined by subtraction of the expected weight of AgI from that of silver halide obtained in a Carius determination. (Found: C, 52·0; H, 6·5; N, 10·6; I, 12·8; Cl, 1·8%. $C_{64}H_{94}O_{12}N_{12}I_{1\cdot6}Cl_{0\cdot4},3H_2O,2C_2H_5.OH$ requires C, 51·6; H, 7·1; N, 10·6; I, 12·8; Cl, 0·9%.)

These analytical results indicate that substitution of chlorine by iodine probably did not exceed 80%. Accordingly in a later preparation, in which the highly purified specimen IV had served as starting material, the treatment with NaI in acetone was done under reflux for 30 min., following Friedman & Rutenburg (1950). However, the I/N ratio in the product was even lower (1·15 atoms of I/12 atoms of N; the I determination was kindly done by Miss B. W. Simpson of the Rowett Research Institute).

Iodine addition compound of N-iodoacetylgramicidin S. On mixing aqueous ethanolic solutions of iodoacetylgramicidin S and iodine an immediate yellow precipitate formed. This could be recrystallized by slow evaporation in air at room temp. from aqueous ethanol or aqueous dioxan to give large red crystals. The product has not been analysed. It was assumed to be a molecular compound with iodine rather than the product of any chemical reaction (cf. Cramer, 1954).

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APPENDIX 2

Possible Molecular Models for Gramicidin S and their Relationship to Present Ideas of Protein Structure

By DOROTHY CROWFOOT HODGKIN and BERYL M. OUGHTON
Chemical Crystallography Laboratory, University Museum, University of Oxford

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The knowledge that has accumulated on the precise structure of amino acids and simple peptides makes it possible to build a number of models which may represent the detailed arrangement of the atoms in gramicidin S. These models must be judged in relation to the evidence described that the molecule behaves as a decapeptide in which two pentapeptide halves are related by a twofold axis of symmetry, and that the atoms tend to be concentrated in layers 4·8 Å apart in a direction at right angles to this twofold axis. In addition, the results of the studies made by Abbott & Ambrose (1953) of the infrared spectra of gramicidin S crystals have to be considered.

Abbott & Ambrose (1953) showed that, in the two main series of crystals examined, hexagonal *N*-acetylgramicidin S and orthorhombic gramicidin S hydrochloride, the infrared dichroism of the crystals is strong and is consistent with an orientation of the CO and NH bonds not far from parallel with the *c* axis, i.e. normal to the strongly reflecting planes of spacing 4·8 Å. Further, they favour a folded α-chain configuration on the evidence that the frequency of the C=O stretching mode in gramicidin S is 1646 cm.$^{-1}$, similar to that of α- and not β-keratin, evidence which is supported by observations on the dichroism of the absorption bands in the 4500–5000 cm.$^{-1}$ region. These observations suggest that we should concentrate our model-building on chain configurations which have been put forward for α-folded proteins, particularly those related to the Pauling, Corey & Branson (1951) α-helix and similar helices. But since there is some doubt about the reliability of conclusions based on small shifts in the frequencies of spectral lines (Ehrlich & Sutherland, 1953), we have thought it best to include also models based on extended β-chain configurations, while accepting the evidence of Abbott & Ambrose (1953) on the general orientation of the CO and NH groups in our crystals. In our model-building we have not considered the possibility put forward by Gavrilov and others (Silaev, Trefilova & Ioanisiani, 1951; Ioanisiani, Gavrilov & Plekhan, 1954; Akimova & Gavrilov, 1954; Reznichenko, 1954) that the peptide structure of gramicidin S may be modified chemically by internal condensation between certain carbonyl and imino groups. None of our data at present are sufficiently precise to exclude such a condensation, but it would be incompatible with most of the models we have so far built.

Within the framework of a simple peptide molecule the following models appear to us most worthy of serious consideration.

α_{11} *Ribbon*. Abbott & Ambrose (1953) have put forward a decapeptide model for gramicidin S based on the Huggins (1943) α_{11} ribbon configuration. Their particular version has not a strict twofold axis of symmetry; it is possible, however, to build a model on this configuration which has the required symmetry. It is illustrated in Fig. 1 and is composed of two four-residue α_{11} ribbons joined at the two ends by the remaining two residues. The rupture of the hydrogen-bonding system at the two proline residues caused by the eclipse of the NH groups allows freer rotation at these two points, and the two halves of the ring can be so oriented that they are related exactly by a twofold axis of symmetry passing through the centre of the ring.

The configuration starting at the α-carbon of a proline residue and working round the ring is as follows:

CR *cis* to NC_δ } proline residue
CO *cis* to CC_β }

CR *cis* to NH } valine residue
CO *cis* to CR }

CR *cis* to NH } ornithine residue
CO *cis* to CR }

CH *cis* to NH } leucine residue
CO *cis* to CH }

Intermediate between
CR *trans* and CH *cis* to NH } phenylalanine residue
CR *cis* and CH *trans* to CO }

This molecular model is sterically satisfactory and will fit into the crystal with the CO and NH bonds approximately parallel with the *c* axis. But it is difficult to see how it could give rise to the observed strong 4·8 Å reflexions, as the repeat distance between residues along the chains is 2·75 Å (Donohue, 1953).

α-Folded chain models based on the α-helix. If moves of the type used in constructing the α-helix of Pauling *et al.* (1951) are made, the five residues of gramicidin S link most naturally into a single pentapeptide ring, with all CO groups projecting from one side of the ring and all NH groups from the other (Fig. 2). Rings of this kind may be piled one above the other in the crystal lattice to form a completely hydrogen-bonded column with atoms in layers about 4·8 Å apart and CO and NH bonds nearly parallel with *c*.

In this otherwise attractive structure the pentapeptide molecules within one column cannot be exactly related by twofold axes of symmetry. It would be possible in the hexagonal crystals to relate two-thirds of the columns in pairs by symmetry axes, leaving the remaining third to be disordered, alternately pointing up and down; or one might perhaps suppose that pentapeptide rings of opposite sense were related within the columns by approximate but not exact twofold axes. We are doubtful whether at the present stage of our investigation we could exclude this type of solution on X-ray evidence alone. But it does appear to be excluded by other evidence mentioned above, and particularly by the reported synthesis of the peptide.

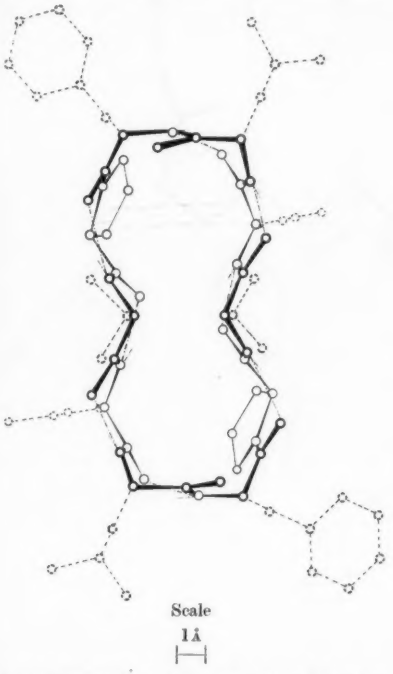

Fig. 1. A decapeptide model for gramicidin S based on the Huggins α_{11} ribbon configuration, viewed down the twofold axis.

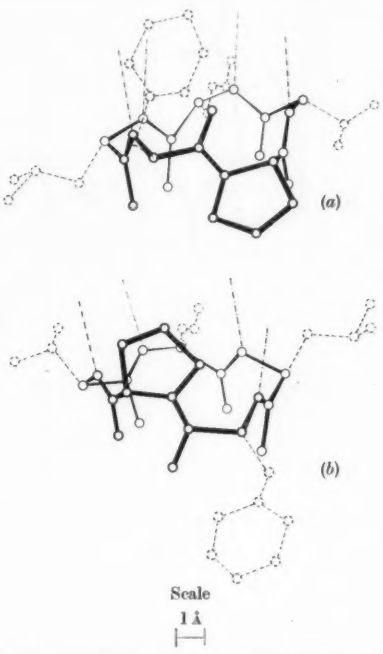

Fig. 2. Pentapeptide models for gramicidin S based on the moves in: (*a*) a left-handed α-helix; (*b*) a right-handed α-helix.

An alternative possibility within the framework of the α-helix configuration is to form a true cyclic decapeptide by breaking the two pentapeptide rings between the α-carbon of the D-phenylalanine and the CO of the proline residue and rejoining to form a horseshoe-shaped molecule. One join is made easily, the other only with distortion of the hydrogen-bonded system, and again the molecule has at best only a pseudo-twofold axis of symmetry. This molecule has packing advantages similar to those of the pentapeptide units, advantages which are not shown by other types of model we have tried to construct in the α-helix configuration; in other respects it is an awkward object.

β-Chain models. By far the most obvious type of model to build for gramicidin S is one based on the extended β-form of chain. Two chains, each composed of four residues, may be set antiparallel to one another and linked at the ends by means of the proline residues to form a decapeptide ring. The chain configuration may be made to conform closely with that of the β pleated sheet of Pauling & Corey (1951). Four hydrogen bonds are formed between the two antiparallel chains, while the remaining CO and NH groups are available for hydrogen bonding between molecules. Several variants are possible, of which one is shown in Fig. 3.

This model is of a decapeptide with a twofold axis of symmetry. In the crystal, molecules could be arranged with their lengths at right angles to c and approximately 4·8 Å apart, the CO and NH bonds projecting parallel with *c*. There are certain

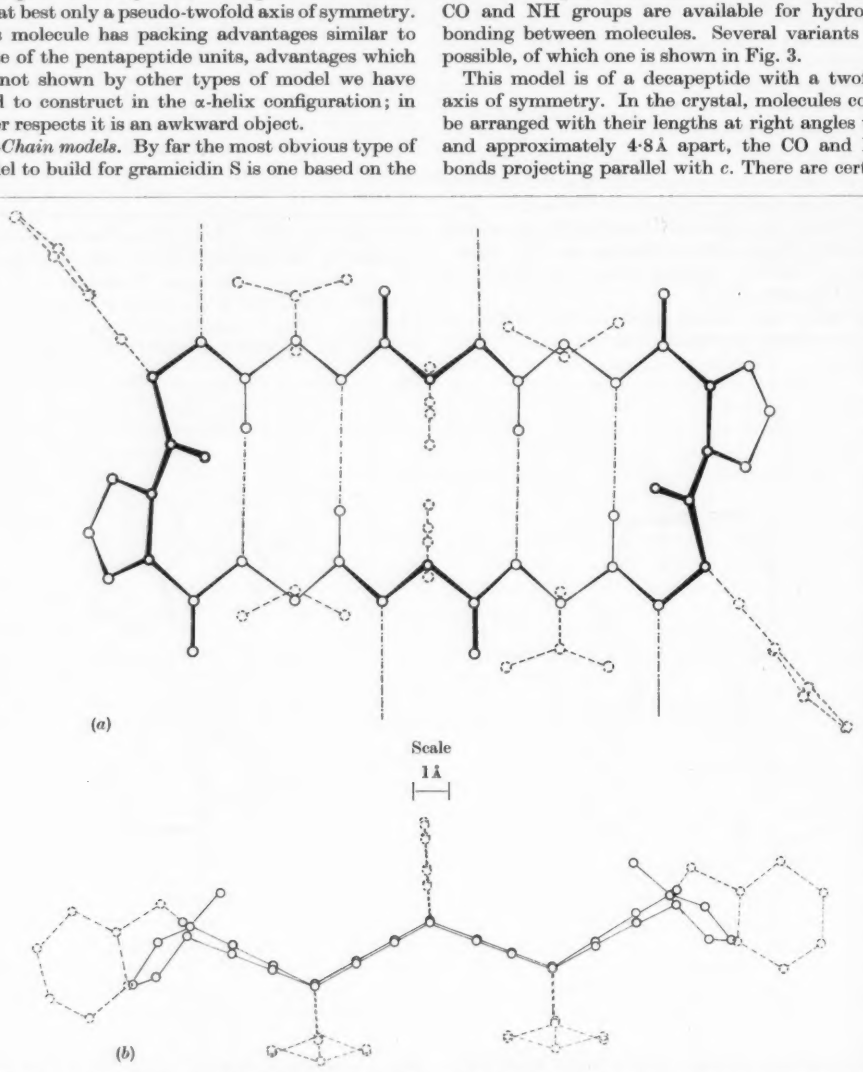

Fig. 3. A decapeptide model for gramicidin S based on the β-pleated sheet of Pauling & Corey (1951) viewed (*a*) down the twofold axis, and (*b*) at right angles to the twofold axis.

problems of intermolecular hydrogen bonding between molecules of this type in the hexagonal crystals which may be surmounted by the intervention of water or ethanol molecules. Otherwise the only immediate serious argument against this model is the optical evidence in favour of an α-type configuration.

Mixed α–β model. A last model which may be mentioned can be built by following partly α, partly β moves (Fig. 4). These may be described as follows: (*a*) valine to ornithine, α-type move (the NH bonds point in the same direction, and perpendicular to the plane of the ring); (*b*) ornithine to leucine, β-type move (the NH bonds point in opposite directions); (*c*) leucine to phenylalanine, α-type move. This model can be formed either as a pentapeptide or as a decapeptide, and appears to be stereochemically satisfactory in either form. In packing it is very similar to the α-helix models described above, but has a true twofold axis of symmetry. Also the formation of the decapeptide need involve no strain.

We have, in addition, considered a number of other models, one, for example, based on *cis* orientation of the CO and NH groups, but none of these seem so satisfactory from a crystallographic point of view as the last two varieties mentioned here. Of these two, the model based on the β configuration seems on our evidence alone to be by far the more likely.

If gramicidin S has a structure of this type a great deal of reinterpretation of recent data on peptide-chain configuration in proteins will be necessary. Infrared studies have indicated a strong correlation between the modified frequency of certain C=O and N–H modes of vibration observed for different 'α-type' proteins. The characteristics of the X-ray photographs of these proteins have in turn been correlated with the type of diffraction pattern given by an α-helical chain configuration. We are led by our observations on gramicidin S to consider either that the frequencies of the infrared vibration spectra cannot be as exactly correlated with chain configuration as has been supposed, or that the particular mode of chain folding indicated for gramicidin S may occur in some 'α' folded proteins. There are observations on at least one protein, insulin (Ambrose & Elliott, 1951), which underline the same conflict and indeed support both these solutions. Insulin is transformed very readily and reversibly into fibrous insulin (Koltun, Waugh & Bear, 1954). It is difficult to imagine that the change involves a

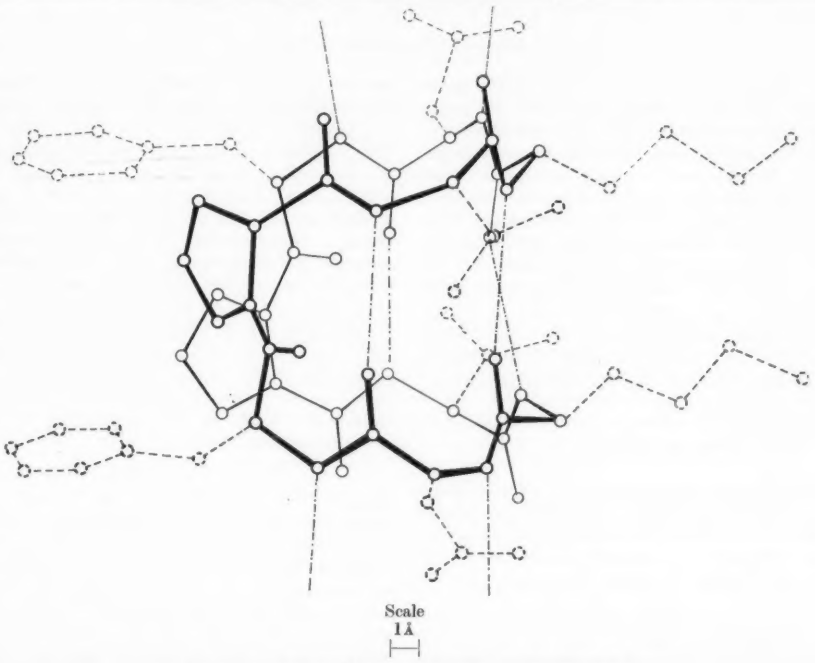

Fig. 4. A mixed α–β decapeptide model for gramicidin S.

major alteration of chain configuration and yet shifts occur in the frequencies of the CO and NH bonds similar to those expected for the change from α- to β-type chain configurations. Further, chain-folding of the type indicated by the model based on the β configuration may well occur in at least part of the insulin molecule (Hodgkin & Oughton, 1956). Unlike the α-helix, this type of folding would permit very easy formation of S–S links between cysteyl residues placed five residues apart in a polypeptide chain, exactly the interval found by Sanger, Smith & Kitai (1954) in the A chain of insulin.

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The Ionization of Acidic Metmyoglobin

IONIC-STRENGTH CHARGE EFFECTS

BY P. GEORGE* AND G. I. H. HANANIA†

Department of Colloid Science, University of Cambridge

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We have shown in a previous publication [George & Hanania, 1952, subsequently referred to as paper (1)] that measurements of the equilibrium concentrations of acidic and alkaline metmyoglobin (MetMb), under conditions of very low ionic strength, I, yield a constant value for the ionization constant only within a narrow range of pH from about 8·6 to 9·3. In more alkaline solutions progressively higher pK values were obtained, and the trend in more acidic solutions was towards lower pK values.

From determinations of the mean pK values between pH 8·6 and 9·3 at very low ionic strengths, the limiting slope of a plot of pK against $\sqrt{I}$ showed that the ionization, which is believed to be that of a water molecule co-ordinated to the haemin iron,

$$Fe^{+}(H_2O) = FeOH + H^{+},$$

is accompanied by the change from -2 to -3 in the effective charge on the iron. The effective charge determined in this way has been called the ionic-strength charge Z (cf. Barnard, 1944). However, the formal charge on the ferric iron atom is not -2 but $+1$, since two of its three positive charges can be regarded as neutralized by negative charges on two pyrrolic nitrogen atoms of the porphyrin ring. This difference is not altogether unexpected, since the ionization occurs about two pH units on the alkaline side of the isoelectric point of myoglobin, and the net negative charge on the molecule, which the acid–base titration curve suggests is about 10 (George & Irvine, 1955), may be considered to influence the reactions of the haemin iron.

Our studies of the formation of metmyoglobin fluoride (George & Hanania, 1954*a–c*) substantiated this idea, for in the pH range 6·5–7·5, which spans the isoelectric point, the change in Z was found to be approximately equal to the corresponding change in formal charge, $+1$ to 0, according to the equation

$$Fe^{+}(H_2O) + F^{-} = FeF + H_2O.$$

That is, somewhere between pH 7·5 and 8·6 the ionization of acidic groups on the molecule alters the value of Z for MetMb from $+1$ to -2. This in turn suggests that the variation of pK' with pH, at low ionic strengths and outside the range of pH 8·6–9·4, might well be due to changes in Z brought about by the ionization of these groups. We present below some detailed measurements made to test this hypothesis.

* Present address: Department of Chemistry, University of Pennsylvania, Philadelphia.

† Present address: Department of Chemistry, College of Arts and Sciences, Baghdad, Iraq.

MATERIALS AND METHODS

Preparation of metmyoglobin. The preparation from horse hearts was carried out as described by George & Irvine (1952) with the following slight modifications. Instead of being taken up finally in a pH 5·7 buffer, the solid MetMb was dissolved in a small quantity of NaCl solution (1%, w/w); Seitz filtration was omitted, and the solutions were stored in sealed ampoules at $-10°$ rather than at 0°. This treatment produced little or no denaturation, and the characteristic extinctions were reproducible to within 1% over a period of many months. When required, portions of the stock were exhaustively dialysed and kept in water at 0°.

The concentrations of all MetMb solutions are expressed in terms of their haematin content and refer to a molecular weight of 17000. However, it should be pointed out that in paper 1 concentrations of MetMb solutions were based on the standards of Duve (1948), but assuming a molecular weight of 17000 instead of the value 16200 used by Duve. Concentrations determined in this way were therefore low by about 4%, and the extinction coefficients were correspondingly high.

Theorell & Åkeson (1955) have recently described an electrophoretic separation of MetMb into three fractions, the main fraction normally accounting for approximately 80% of the haemoprotein. Their method of preparation differs from ours in omitting the treatment with 3M phosphate buffer, pH 6·8. Although simple paper electrophoresis had indicated that the present preparations were homogeneous, it is nevertheless possible that these were also mixtures and the question arises as to what effect this would have on results obtained with them. First, the concentration determined as haematin iron via the pyridine haemochromogen is not affected, but the extinction coefficients and isosbestic points quoted below may be average values. However, we have found these values to be reproducible to within 1% for a number of preparations, which suggests that if several myoglobins are present the same mixture is obtained each time as far as can be judged from spectroscopic characteristics. Secondly, in our previous study in paper 1, the result that $n=1$ for the ionization between pH 8·6 and 9·3 over the entire ionic-strength range indicates that, within experimental error, all the iron in the metmyoglobin shows the same chemical affinity. For this reason we believe that thermodynamic data obtained with the present preparations are truly representative of myoglobin, and that if several myoglobins are actually present the different protein components only influence very slightly the reactivity of the haemin iron atom. The data for the ionization even of metmyoglobin and methaemoglobin are quite similar, as shown in Table 1, whereas there are far greater differences between these two proteins, e.g. solubility, than there are between the three myoglobin fractions.

Spectrophotometric measurements. These were carried out as described in paper 1 with a Unicam quartz spectrophotometer and a water-circulating device for controlling temperature in the cell compartment to $\pm 0·1°$. The absorption spectra of acidic and alkaline MetMb were carefully mapped out from 950 to 240 mμ., and the positions of the bands and their corresponding molar extinction coefficients are given in Table 2. Isosbestic points were located at the following wavelengths: 858, 675, 626, 523, 494, 419 and 269 mμ.

For the purposes of measurement, weakly buffered MetMb solutions about 50 μM were used. The wavelength 584 mμ. was chosen for comparing optical densities, and all measurements were made in 10 mm. glass cells, against water as a blank.

Buffer solutions. These were prepared as described in paper 1. For the pH range 8–10 borate buffers were made from mixtures of H_3BO_3 and NaOH, and for lower pH values mixtures of $NaH_2PO_4,2H_2O$ and NaOH were used. All chemicals were of A.R. quality, and the water was distilled and then passed through a column of exchange resin. Ionic strengths were reckoned on the basis of contributions from the buffer ions, and were adjusted by the addition of the requisite small quantities of NaCl.

Measurement of pH. This was carried out as described in paper 1. The suggestions of Bates, Pinching & Smith (1950) were closely followed with regard to the standardization of the pH scale, and no further corrections were applied for the change in liquid-junction potentials with the changing ionic strength of the solutions.

RESULTS

By the procedure described in paper 1, values for the ionization constant were calculated from the measured pH of the solution and the spectrophotometrically determined ratio of the concentrations of acidic MetMb (C_{HP}) and alkaline MetMb (C_P), i.e.

$$pK' = pH + \log C_{HP}/C_P.$$

In the present study, measurements were extended over a much wider pH range, about pH 7–10, and were made at eight ionic strengths of the buffer ranging from 0·40 to 0·002. The data at three ionic

Table 1. *Thermodynamic data for the ionization of metmyoglobin and methaemoglobin at* 20° *(George & Hanania,* 1952, 1953)

	$\Delta G°$ (kcal. mole^{-1})	ΔH (kcal. mole^{-1})	$\Delta S°$ (cal. mole^{-1} degree^{-1})
MetMb	12·1±0·04	5·75±0·67	−21·7±2·4
MetHb	11·9±0·03	3·91±0·49	−27·2±1·8

Table 2. *Millimolar extinction coefficients* ($10^{-3}\epsilon$) *of acidic MetMb at* pH 6·0 *and alkaline MetMb at* pH 11·0, *corresponding to band-absorption maxima of wavelength* λ

λ (mμ.)	823	633	594	584	543	503	413_5	409_5	280
Acidic MetMb	—	3·52	—	2·66	—	9·18	—	166	32·2
Alkaline MetMb	0·75	—	8·50	8·50	8·80	—	91·0	—	34·9

Table 3. *Values of* pK' *for the ionization of acidic MetMb calculated from equation 2 of paper* 1, *at various* pH *values and at three ionic strengths*

Values in middle section* are from paper 1, and cover the pH range from about 8·5 to 9·0. $T = 20°$.

$I = 0{\cdot}40$		$I = 0{\cdot}01$		$I = 0{\cdot}002$	
pH	pK'	pH	pK'	pH	pK'
7·56	8·99	7·73	8·83	7·36	8·76
7·80	8·98	8·10	8·87	8·04	8·85
8·01	8·99	8·30	8·89	8·28	8·91
8·18	8·99	8·44	8·89	8·43	8·91
8·5*	8·98 ± 0·01	8·5*	8·89 ± 0·01	8·5*	8·93 ± 0·02
9·0*		9·0*		9·0*	
9·53	8·99	9·32	8·88	9·07	8·93
9·63	8·98	9·52	8·90	9·19	8·95
9·76	8·97	9·70	8·92	9·31	8·95
		9·80	8·94	9·43	8·98
				9·60	9·01

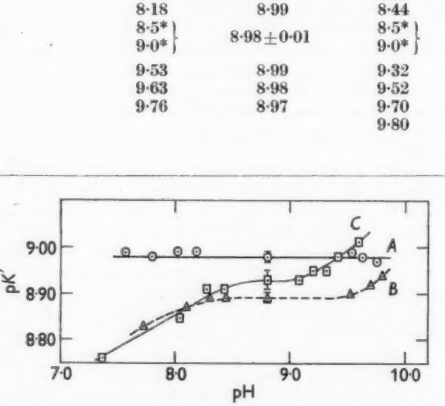

Fig. 1. Values from Table 3 are plotted to show the constancy of pK' with pH at high ionic strengths, $I = 0{\cdot}40$ (A), and the progressive spread of pK' values over a pH range at constant I in solutions of decreasing ionic strength, $I = 0{\cdot}01$ (B) and $I = 0{\cdot}002$ (C). The values taken from paper 1, with their limits of error, are plotted at pH 8·8.

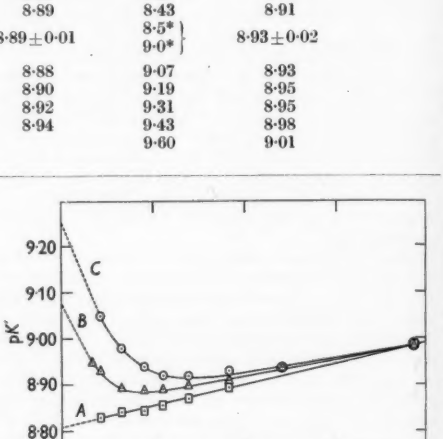

Fig. 2. Variation of pK' at 20° with ionic strength at pH 7·8 (A), at pH values between 8·5 and 9·0 (B), and at pH 9·8 (C), obtained by interpolation (at constant pH) from curves of the type shown in Fig. 1. Curve B is taken directly from paper 1. The limiting linear slopes are approximately $+0{\cdot}5$, $-2{\cdot}5$ and $-4{\cdot}5$ in A, B and C respectively.

strengths, the two extreme values and an intermediate one, are given in Table 3 and are plotted in Fig. 1.

The results are in good agreement with those of paper 1, and they confirm the previous conclusion, namely that the ionization constant is independent of pH only in the presence of appreciable concentrations of a neutral salt. Thus at $I = 0{\cdot}40$ (at 20°) the mean value of pK' is $8{\cdot}98 \pm 0{\cdot}01$ throughout the range of pH 7·5–9·8. The value of pK' is also constant with pH at lower salt concentrations until about $I = 0{\cdot}02$, when a definite spread in the values becomes noticeable, both below pH 8 and above pH 9. The spread becomes progressively greater at lower ionic strengths and, as can be seen in Fig. 1, it is quite marked at $I = 0{\cdot}002$.

The effect is better illustrated when the data are plotted at constant pH. By interpolating between experimental values of pK', and in one case by using a slight extrapolation, two sets of pK' values as a function of ionic strength were obtained, at pH 7·8 and 9·8. The plots are shown in Fig. 2, which also includes the curve at 20° from paper 1. Extrapolation of these curves to $I = 0$, assuming that they are linear at very low ionic strength, gives the approximate pK_0 values of 9·25 at pH 9·8 and 8·81 at pH 7·8, which are to be compared with the value 9·07 from paper 1 obtained in the intermediate pH range. Likewise the approximate limiting slopes of the lines are about $-4{\cdot}5$ at pH 9·8 and $+0{\cdot}5$ at pH 7·8, as compared with $-2{\cdot}5$ in the intermediate range.

DISCUSSION

The above results and similar data may be used to furnish a simple and semi-quantitative measure of the influence of a protein on the reactivity or affinity of its active site. First, however, it is necessary to show that this remarkable effect of pH on the ionization of acidic MetMb is a genuine effect of the ionization of acidic groups on the free energy of ionization of the iron-bound water molecule, and

is not due merely to shifts in the position or intensity of the absorption bands of acidic or alkaline MetMb at very low ionic strengths.

The likelihood that this is a genuine chemical effect may be inferred from a comparison between methaemoglobin (MetHb) and MetMb. In the ionization of acidic MetHb, there is a slight shift in the absorption spectrum of the conjugate acidic form at very low I (George & Hanania, 1953), and as a result of this the apparent ionization constant pK' changes in a uniform manner over the whole pH range 8–10 at low I, the effect becoming progressively more pronounced at lower values of I. However, the ionization constant of MetMb calculated from measurements between pH 8·6 and 9·3 is constant even at the lowest values of I, and so the factor which makes pK' a variable in the ionization of MetMb cannot be the same (spectroscopic) factor operative with MetMb. Likewise it can be shown that a similar shift in the absorption spectrum of alkaline MetMb would also effect pK' throughout the entire pH range, and not merely outside the range 8·6–9·3.

It seems reasonable therefore to conclude that the above results arise from a genuine chemical effect. On this basis, following the argument used in paper 1, we may take a simple Debye–Hückel model for calculating the effective (ionic strength) charge, Z, on the haematin iron atom from the limiting slopes in Fig. 2. To the nearest whole number, we find that upon ionization at pH 7·8 Z changes from $+1$ to 0, whereas between pH 8·6 and 9·3 it changes from -2 to -3, and at pH 9·8 from -4 to -5.

The corresponding change in the formal charge on the iron atom accompanying the ionization of acidic MetMb is $+1$ to 0 in every case. That is, the effective charge on the iron atom in acidic MetMb is like the formal charge, $+1$, at pH 7·8 even in solutions of extremely low I. However, in more alkaline solutions the value of Z changes progressively to -2 at pH 9 and to -4 to pH 9·8, and the general trend in the results suggests that this value could well rise above -4 in still more alkaline solutions.

Can the equivalent of five negative charges be produced between pH 8 and 10? The acid–base titration curve of MetMb shows that the net negative charge on the molecule is about 10 at pH 9 and about 20 at pH 10 (George & Irvine, 1955), but it is not clear how the electrostatic effect of these groups is transmitted to the haematin iron. We have previously suggested that a distinction can be made between this effect and the well-known 'haem-linked effect' (George & Hanania, 1954*c*). With MetMb there is a clearly marked haem-linked effect which can be accounted for adequately in terms of one acidic group with $pK' \approx 6{\cdot}0$ at 20°, and which will therefore be almost entirely in its conjugate basic form in solutions alkaline to pH 8 (George & Hanania, 1955). Moreover, this effect is operative at high as well as low I. On the other hand, the phenomenon described in this paper appears only at very low I, and is completely suppressed by salt concentrations whose ionic strength exceeds 0·1. Accordingly, whatever the chemical nature of these groups, they must be regarded as having no special stereochemical configuration or chemical bonding relative to the haematin iron atom. The possible role which cationic acids may play in producing this effect has been discussed in some detail (George & Hanania, 1954*c*).

As to the magnitude of this effect, it can be seen from Fig. 2 that the maximum difference between pK' values is at $I = 0$, and is 0·44 unit at 20° between pH 7·8 and 9·8. In terms of free energy this amounts to about 0·6 kcal./mole. Since the standard free energy of ionization of acidic MetMb is 12·1 kcal./mole at 20°, this effect is seen to alter the free energy by about 5 % at its maximum, and it may therefore be described as a second-order effect. Nevertheless, this indicates the extent to which ionizations on the protein influence the acid–base equilibrium of the iron-bound water molecule.

There are severe complications in carrying out a similar investigation of the methaemoglobin ionization, notably the change in the absorption spectrum of the conjugate acid at low I, but a rough estimate of the corresponding effect may be made from a comparison between the 'critical' ionic strengths in MetMb and MetHb, i.e. the values of I at which the pK'–$\sqrt{I}$ curve changes the sign of its slope. Spectrophotometric studies of the ionization of acidic MetHb (George & Hanania, 1953) showed that the critical I for MetHb is about 0·1 compared to 0·01 for MetMb, and that the limiting slopes of their pK'–$\sqrt{I}$ plots in the pH range 8·6–9·3 are about equal. If it is assumed that the behaviour of the two haemoproteins is similar, i.e. with the same limiting slopes at pH 7·8 and at pH 9·8, then the maximum difference in pK between pH 7·8 and 9·8 in the ionization of MetHb would be about three times the difference found with MetMb, and the corresponding difference in free energy would be about 1·9 kcal./mole. At 20°, the standard free energy of ionization of acidic MetHb is 11·9 kcal./mole; hence the free energy would be altered by about 16 % at its maximum, which is considerably greater than with MetMb. Thus we may conclude that in the MetHb ionization, with its larger critical I, there might be a bigger difference in pK_0 values between pH 7·8 and 9·8, which would be attributable to a stronger interaction between the haematin iron atom of MetHb and ionizing groups on the protein.

A similar conclusion follows from the assumption that the critical I represents the point of departure from simple Debye–Hückel behaviour at the

particular pH owing to the screening of the haematin iron atom from the influence of the various ionizing groups. On this basis a larger critical I would signify that a larger concentration of salt ions is required for complete screening, and hence that there is a stronger interaction between the iron and the ionizing groups in MetHb.

In conclusion, although the mechanism may be different it may be noted that the Bohr effect, which is due to interaction between the iron and the specific haem-linked ionizing group, is greater in haemoglobin than in myoglobin.

SUMMARY

1. The ionization constant of the iron-bound water molecule in acidic metmyoglobin has been measured from about pH 7 to 10 at eight ionic strengths of buffer solution ranging from 0·40 to 0·002. The values confirm the conclusion suggested by the measurements reported in paper 1 (George & Hanania, 1952) that the ionization constant is independent of pH only in the presence of appreciable concentrations of neutral salt.

2. At $I = 0{\cdot}40$ at 20°, pK' is 8·98 throughout the pH range 7·5–9·8, but at $I < 0{\cdot}02$, pK' decreases as pH is decreased below 8·6, and it increases as pH is increased above 9·3.

3. The extrapolation of pK' values plotted against $\sqrt{I}/(1+\sqrt{I})$ to zero ionic strength gives pK_0 values of 8·81, 9·07 and 9·25 at pH 7·8, between pH 8·6 and 9·3 and at pH 9·8 respectively.

4. It is suggested that this variation of the free energy of ionization with pH is due to an unspecific interaction between charged groups on the protein moiety and the haematin iron atom, which is observable only at low I because of the screening effect of neutral salt ions. In contrast, the effect of a specific haem-linked ionizing group is observable at all ionic strengths.

5. The limiting slopes of the pK' plots are approximately $+0{\cdot}5$, $-2{\cdot}5$ and $-4{\cdot}5$ at pH 7·8, between pH 8·6 and 9·3 and at pH 9·8, which, on the basis of a simple Debye–Hückel model may be attributed to the effective charge of the haematin iron atom changing upon ionization from $+1$ to 0, from -2 to -3 and from -4 to -5 respectively.

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The Sulphydryl Groups of Sickle-cell Haemoglobin

By V. M. INGRAM
Medical Research Council Unit for the Study of the Molecular Structure of Biological Systems, Cavendish Laboratory, University of Cambridge

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In patients with homozygous sickle-cell anaemia the red blood corpuscles become distorted into sickle shapes at low oxygen pressure, apparently due to the formation of liquid crystals of reduced haemoglobin. Such liquid crystals can also be formed *in vitro* by reduction of purified solutions of sickle-cell haemoglobin, a phenomenon which is apparently due to the abnormally low solubility of sickle-cell haemoglobin in the reduced state (Perutz & Mitchison, 1950; Perutz, Liquori & Eirich, 1951). The formation of liquid crystals can be inhibited and the solubility restored to normal by reaction with SH group inhibitors such as silver and mercury ions and mercury derivatives (Thomas & Stetson, 1948; Ingbar & Kass, 1951). Presumably the sulphydryl groups are closely involved in the 'sickling' process. Ingbar & Kass (1951) made a study of the number of available SH groups with amperometric silver nitrate titrations and made the significant observation that there were apparently two SH groups in normal but three in sickle-cell haemoglobin (haemoglobin A and S respectively).

Some years later Ingram (1955) studied the SH groups of several species of haemoglobins, including human, by a similar technique and found native human haemoglobin A to have not two, but four, available sulphydryl groups, a number which increased to eight on denaturation with sodium dodecyl sulphate. In view of the contradiction between the two sets of results it seemed desirable to repeat the measurements of Ingbar & Kass on sickle-cell haemoglobin. Furthermore, although Benesch, Lardy & Benesch (1955) found eight SH groups after denaturation with 8 M urea, in agreement with my own results, Hommes, Santema-Drinkwaard & Huisman (1956) found eight SH groups in the native haemoglobins A and S and an uptake of 5·4 and 7·0 molecules of $HgCl_2$ respectively per native haemoglobin molecule, whereas the corresponding numbers found by myself had been four SH groups and 2 molecules of $HgCl_2$ in haemoglobin A. Closely related methods were used by all the different investigators. As this paper shows, I was able to confirm my previous results.

Murayama (1956) has titrated deoxygenated haemoglobins A and S at 0° and found four SH groups, in agreement with the values reported in this paper for oxyhaemoglobins A and S at room temperature. However, at 38° he finds a difference between the two proteins, namely three and two SH groups per molecule of haemoglobin A and S respectively, i.e. results which are the exact opposite to those reported by Ingbar & Kass (1951).

METHODS AND RESULTS

The methods previously described were used with only minor modifications (Ingram, 1955).

Mercurimetric titrations were first performed in 5 ml. of solution with a rotating Pt-wire electrode in 0·05 M-NH_4NO_3 and 0·1 M-NH_3 soln., $Hg(NO_3)_2$ being the titrating agent. The haemoglobin solutions were prepared as previously described and dialysed as indicated in Table 1, which gives the results obtained. Fig. 1 shows examples of the Hg-titration plots. Such end points leave no room for uncertainty about the number of atoms of Hg bound by a molecule of protein, whether native or denatured. No difference could be detected between haemoglobins A and S.

Argentometric titrations were next undertaken, a normal, clean Pt-wire electrode being used. The supporting electrolyte was again 0·05 M-$NH_4(NO_3)$ with NH_3 of the strength indicated in Table 1. These titrations gave lower results

Table 1. *Reactions of metal ions with haemoglobin*

Mercury titrations

	Dialysis	Storage time* (days)	NH_3 concn. (M)	Hg bound/4Fe: Native	Hg bound/4Fe: Sodium dodecyl sulphate denatured (400 moles/4Fe)
Haemoglobin A	Saline	3	0·10	2·2	5·9
	Saline	19	0·10	2·1	5·6
Haemoglobin S	Saline	10	0·10	2·1	6·1

Silver titrations, normal platinum electrode

	Dialysis	Storage time (days)	NH_3 concn. (M)	Ag bound/4Fe: Native	Ag bound/4Fe: Sodium dodecyl sulphate denatured (400 moles/4Fe)
Haemoglobin A	Saline	6	0·03	2·9	—
			0·10	2·4	—
			0·25	1·8	—
Haemoglobin S	Saline	10	0·03	2·8	—
			0·25	1·8	—
			0·10	—	8·2

Silver titrations, mercury-treated platinum electrode

	Dialysis	Storage time (days)	NH_3 concn. (M)	Ag bound/4Fe: 1st end point	Ag bound/4Fe: 2nd end point
Haemoglobin A	Saline	2	0·03	4·2	—†
	Saline	4	0·03	3·0	8·0
	Saline	7	0·03	2·8	7·9
	Water	2	0·03	3·9	—†
	Water	4	0·03	2·9	7·7
Haemoglobin S	Saline	1	0·03	3·8	8·4

* Days counted from thawing the washed and frozen red cells.
† The second end point was not suspected when these titrations were performed, although it was probably present.

than those previously reported, but the samples of both proteins happened to be considerably more than 2 days old. As is shown in the table the number of available SH groups decreased rapidly when the sample was stored, even at 4°. Again normal and sickle-cell haemoglobins gave identical results, showing a similar decrease of detectable SH with rising NH_3 concentration, as reported earlier (Ingram, 1955).

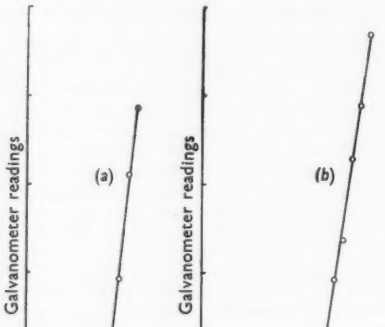

Fig. 1. Titration with mercuric nitrate of (*a*) native haemoglobin, and (*b*) haemoglobin denatured with sodium dodecyl sulphate.

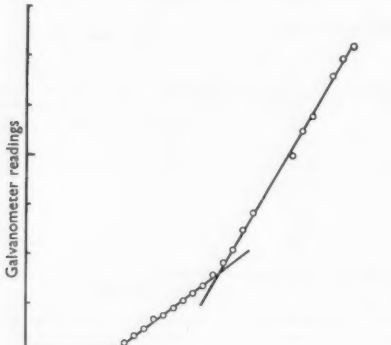

Fig. 2. Titration with silver nitrate of native haemoglobin S. The electrode was treated with ammoniacal mercuric nitrate solution before use.

The finding of eight SH groups in the sickle-cell haemoglobin denatured with sodium dodecyl sulphate agrees with the number found previously for the normal protein.

Hommes *et al.* (1956) had found it necessary to pretreat their electrode by immersing it in an ammoniacal $HgCl_2$ solution. Therefore for the final set of titrations a rotating platinum-wire electrode was used which had previously been employed for $Hg(NO_3)_2$ titrations in dilute NH_3 soln. Such a system did indeed give different titration plots with native haemoglobin when $AgNO_3$ was used; it can be seen that two end points (Fig. 2) were obtained. The table shows that the first end point of four SH groups corresponds very closely to the number of SH groups found in the native protein with the untreated platinum electrode. The second break in the titration curve occurs at 8Ag:4Fe, the number found previously in the denatured haemoglobin. Presumably this is the end point observed by the Dutch workers with their mercury-treated electrode.

In the present experiments fresh sickle-cell haemoglobin has given values for the SH groups which are almost identical with those for the normal protein, but both human haemoglobins show a pronounced storage effect. Frozen solution also deteriorates, but frozen red cells do not, perhaps because the presence of glutathione in the latter has a stabilizing effect. Horse haemoglobin, on the other hand, is quite stable for at least a week at 4°. The change is unlikely to be due simply to the formation of disulphide bonds since the older samples still show eight SH groups when denatured.

DISCUSSION

It has been known since 1949 (Pauling, Itano, Singer & Wells, 1949; Scheinberg, Harris & Spitzer, 1954) that there is a difference in the electrophoretic mobilities, and therefore in the number of charged groups, of normal human and sickle-cell haemoglobin. The only other definite evidence of a chemical difference between the proteins is a change in the amino acid sequence of one small portion of a polypeptide chain (Ingram, 1956).

The most important though disappointing result of the present titration experiments is that the numbers of SH groups in the native and denatured forms of normal and sickle-cell haemoglobin are the same. Shortage of material prevented further examination of the sulphydryl groups of the abnormal haemoglobin by the blocking technique with mercury derivatives (Ingram, 1955). The present results leave no doubt that haemoglobin S shows the same pattern of heavy-metal uptake as normal haemoglobin. The four available SH groups of the native haemoglobin S combine with four silver atoms, but only two mercury atoms. On denaturation with sodium dodecyl sulphate another four SH groups are revealed, making a total of eight, whilst at the same time four additional mercury atoms are bound, making six in all. This similarity in behaviour suggests strongly that the arrangement of these groups, as well as their numbers, is the same in the two haemoglobins, i.e.

two equivalent clusters of three SH groups and a further two SH groups in separate but equivalent positions.

My results agree with those of Murayama obtained at 0°, but not with those of Huisman and his collaborators, except that they also were unable to find a difference between the two haemoglobins. Their figures for the silver and mercury uptake of native haemoglobin A and S are very close indeed to those obtained in the present, and previous, experiments with samples fully denatured by sodium dodecyl sulphate. Their method of preparing the haemoglobin is a different and perhaps a harsher one than the method used in this work; this might explain the high uptake of heavy metal without previous denaturation. With respect to the first of the two breaks in the titration plot obtained with a mercury-coated electrode, it is possible that rather small changes in the titration conditions might make it almost invisible.

SUMMARY

1. The numbers of sulphydryl groups in native and sodium dodecyl sulphate-denatured sickle-cell haemoglobin are the same as in the normal haemoglobin. Their configuration is probably also the same.

2. Two kinds of sulphydryl groups, which have different affinities for silver, can be estimated by amperometric titration with silver nitrate at the mercury-treated platinum electrode estimate. The groups correspond apparently to the sulphydryl groups available to silver at the untreated electode in native and in denatured human haemoglobin.

3. Storage at 4° leads to a loss in sulphydryl groups available in the native protein.

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The Oxidation of Tryptamine to 3-Indolylacetaldehyde by Plant Amine Oxidase

By A. J. CLARKE and P. J. G. MANN
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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3-Indolylacetic acid (IAA) is believed to be a principal auxin of higher plants. There is much evidence that it is formed in plants from tryptophan but the metabolic route is not yet established. It has been suggested that the route is by way of tryptamine and 3-indolylacetaldehyde (IAc) or 3-indolylpyruvic acid and IAc. Two oxidases, or groups of oxidases, catalysing the oxidation of amines have been found in higher plants. The presence of a monoamine oxidase was shown by Werle & Roewer (1950, 1952), and that of a histaminase or diamine oxidase by Werle & Zabel (1948) and Werle & Pechmann (1949). Kenten & Mann (1952*a*) found that the diamine oxidase catalysed the oxidation of both mono- and diamines and preferred the name plant amine oxidase. It was shown that the oxidation of tryptamine is catalysed by the enzyme and this reaction, followed by the action of an aldehyde oxidase on the product, was suggested as a possible mechanism of formation of IAA in higher plants. Subsequent attempts to prove that IAc is the product of the oxidation of tryptamine catalysed by plant amine oxidase, with the relatively crude preparations of the enzyme described by Kenten & Mann (1952*a*), were unsuccessful. In these experiments the observed oxygen uptake was much greater than that required for oxidation of tryptamine to the corresponding

aldehyde, which suggested the presence in the crude enzyme preparations of factors catalysing the oxidation of IAc. Mann (1955) has since purified plant amine oxidase several hundredfold, and the results of an investigation of the oxidation of tryptamine catalysed by such purified preparations of the enzyme are here recorded.

EXPERIMENTAL

Amine oxidase. Purified preparations of amine oxidase were obtained by the method of Mann (1955) with some slight modifications. Pea seedlings (*Pisum sativum* L.) were grown for 8–12 days in sand. The washed cotyledons alone were used as starting material, since Kenten & Mann (1952*a*) have shown that most of the enzyme is present in the cotyledons at this stage of growth. The cotyledons were macerated for 2 min. in an Ato-Mix blender (Measuring and Scientific Equipment Ltd.) with 0·067 M phosphate buffer, pH 7, previously cooled to 0–5° (100 ml./100 g. of cotyledons) and the macerate was squeezed through strong cotton cloth. The residual pulp was mixed with more buffer (50 ml./100 g. of cotyledons) and again squeezed through cloth. The combined extracts were cooled in an ice–salt freezing mixture to between 0 and 5°, and a mixture of ethanol–chloroform (20 ml. of ethanol and 10 ml. of chloroform/100 ml. of extract), previously cooled to −10°, was added slowly during vigorous mechanical stirring. The stirring was continued for a further 90 min. after the addition of the ethanol–chloroform mixture. The subsequent procedure was that of Mann (1955). The volume of the final enzyme solutions was such that 1 ml. was equivalent to 50 g. of cotyledons. About thirty such preparations were used in the work and the N contents and specific activities (Mann, 1955) of seven were estimated. The N contents varied from 425 to 690 μg./ml. with an average of 590 μg./ml. The specific activities varied from 64000 to 114000 with an average of 92000.

Relatively crude dry preparations of amine oxidase were prepared from pea-seedling extracts by fractional precipitation with ammonium sulphate followed by precipitation with acetone, as described by Kenten & Mann (1952*a*). These are referred to in the text as the dry preparations of amine oxidase.

Peroxidase. This was prepared from horse-radish roots by the method of Kenten & Mann (1954), except that the final fractional precipitation with ethanol was omitted. The Purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the preparation, i.e. mg. of purpurogallin formed by 1 mg. of enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was estimated by the method of Keilin & Hartree (1951) with an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622 (maximum transmission at λ465 mμ.). The preparation of P.Z. 900 used was about 80% peroxidase.

The peroxidase activities of some of the amine oxidase preparations were also estimated by the same method.

Catalase. This was prepared from ox liver by the method of Sumner & Dounce (1937). The activity of the preparations was estimated as described previously (Kenten & Mann, 1952*b*). The Katalasefähigkeit (Kat. f.) values of the two preparations used were 11000 and 27000, suggesting purities of about 20 and 50% respectively. From the equation of Chance & Herbert (1950) that Kat. f. = 520 k_1'/M, where M is the molecular weight of catalase (230000), the velocity constants of the preparations are:

$$k_1' = 4{\cdot}9 \times 10^6\,\text{M}^{-1}\,\text{sec.}^{-1} \quad \text{and} \quad 11{\cdot}9 \times 10^6\,\text{M}^{-1}\,\text{sec.}^{-1}.$$

In experiments with added catalase, 100 μg. of the preparation of Kat. f. 11000 or 50 μg. of that of Kat. f. 27000 were used/3 ml. of reaction mixture.

Notatin. This was a preparation given by the Boots Pure Drug Co. in 1952.

Buffers. Phosphate buffers (0·2 M) were prepared from solutions of KH_2PO_4 and KOH. Pyrophosphate buffers (0·2 M) were prepared from solutions of $Na_2H_2P_2O_7$ and $K_4P_2O_7$. Phosphate–borate buffers were made by mixing equal volumes of 0·4 M-KH_2PO_4 and 0·4 M-H_3BO_3 and adjusting to the required pH with KOH, finally diluting to twice the original volume.

Ammonia. This was estimated by diffusion in Conway dishes at room temperature.

IAA. 3-Indolylacetic acid was estimated by means of the Salkowski reaction. Houff, Hinsvark, Weller, Wittwer & Sell (1954) have shown that this reaction, the formation of a red colour when IAA reacts with Fe^{3+} ions in acid solution, depends on the oxidation of IAA to *N*-hydroxy-IAA. The estimation procedure used in the present work was one of those suggested by Gordon & Weber (1951). Samples of the reaction mixtures, preferably containing 20–70 μg. of IAA, were diluted to 10 ml. with water and 15 ml. of modified Salkowski reagent (a mixture of 5 ml. of 0·5 M-$FeCl_3$, 250 ml. of water and 150 ml. of conc. H_2SO_4) was added. The solutions were thoroughly mixed and allowed to stand at least 1 hr., and not more than 4 hr., at room temperature. The optical densities at 530 mμ. were then measured in matched tubes in a Unicam model SP. 400 spectrophotometer. Samples of reaction mixtures without added tryptamine were treated with the reagent in the same way and used in the blank tube. The calibration curve obtained with IAA, a mixture of 10 ml. of water and 15 ml. of Salkowski reagent being used in the blank tube, was almost linear with amounts of IAA up to 70 μg. The optical density at 530 mμ. reached a maximum in about 45 min. and little difference was found in the calibration curve whether the time allowed for colour development was 1 or 4 hr. These results agree with the results of Gordon & Weber (1951).

IAc. 3-Indolylacetaldehyde was oxidized with silver oxide to IAA, which was then estimated spectrophotometrically with Salkowski reagent. Oxidation with silver oxide forms the basis of several methods of estimating aldehydes but some difficulty was experienced in adapting the existing oxidation procedures. Separately prepared silver oxide, even when freshly precipitated, gave erratic results and the method finally evolved was derived from those of Pondorff (1931) and Siegel & Weiss (1954) in which the silver oxide was precipitated in the reaction mixtures. Samples of the reaction mixtures, not more than 0·5 ml. and preferably containing 100–300 μg. of IAc, were diluted with water to 1 ml.; 1 ml. of 0·1 M-$AgNO_3$ was then added, followed by 1 ml. of 0·1 M-NaOH and, lastly, 1 ml. of 0·2 M phosphate, pH 7. When a series of estimations were being done simultaneously the phosphate was added immediately after the NaOH. The solutions were thoroughly mixed after each addition and finally shaken mechanically for 5 min. in darkness. They were then filtered through Whatman no. 44 filter papers and 1 ml. samples of the filtrates were used for

the estimation of IAA. Control reaction mixtures without added tryptamine were treated with silver oxide in the same way and the filtrates with added Salkowski reagent were used in the blank tube in the IAA estimations.

Manganese. After wet digestion of enzyme samples the manganese present was oxidized with potassium periodate to permanganate, which was estimated colorimetrically.

Absorption curves. These were determined in a Unicam model SP. 500 spectrophotometer with 1 cm. cells.

Paper chromatography. A descending one-dimensional technique was used with Whatman no. 4 paper. The solvent was a mixture, *iso*propanol–NH_3 soln. (sp.gr. 0·880)–water (10:1:1, by vol.), as used by Bennet-Clark, Tambiah & Kefford (1952) and Stowe & Thimann (1954). The papers were dried at room temperature and sprayed with Ehrlich reagent (2 g. of *p*-dimethylaminobenzaldehyde in a mixture of 80 ml. of ethanol and 20 ml. of conc. HCl).

Manometric methods. Measurements of O_2 uptake were made, in air, in the Warburg apparatus at 28°. The volume of the reaction mixtures was usually 3 ml., and 0·2 ml. of 5N-KOH was present in the centre well unless otherwise stated. In a few experiments 9 ml. reaction mixtures were used in large Warburg vessels of total volume about 60 ml. Formation of CO_2 was measured by Warburg's direct method (Dixon, 1943).

Melting points. These are uncorrected.

RESULTS

Manometric studies of the oxidation

Effect of pH. The variation of activity with pH was tested in reaction mixtures containing amine oxidase, catalase and tryptamine (0·0067M) in phosphate–borate buffers, pH 6–10. Mann & Smithies (1955) showed that, at least over the pH range 7–8, borate does not inhibit plant amine oxidase. The pH–activity curve (Fig. 1) was obtained by plotting the O_2 uptakes in the first 10 min. against the final pH. The curve shows an optimum at about pH 8·1, but very little change in activity was found over the range of pH 7·9–8·4. The activity at pH 7 was about half that at the optimum.

Effect of substrate concentration. The effect of varied substrate concentration on the rate of oxidation was tested in the presence of catalase, in phosphate buffers at pH 7 and 8. At pH 8 the maximum initial rate of O_2 uptake was reached at about 0·004M tryptamine. Thereafter further increases in substrate concentration up to 0·02M produced little or no change in the rate. At pH 7 the initial rate of oxidation increased rapidly as the substrate concentration was raised to 0·004M, but thereafter the rate continued to increase, though relatively slowly, as the substrate concentration was raised to 0·02M.

Whereas at pH 8 the initial rate of oxidation is independent of change in substrate concentration from 0·004 to 0·02M, inhibition or inactivation of the enzyme is more rapid at the higher concentrations and, in consequence, the total O_2 uptake may decrease with increasing substrate. Thus the results of Fig. 2 show progressively decreasing total O_2 uptakes by reaction mixtures at pH 8 as the initial tryptamine concentration was raised from 0·0033 to 0·02M. Similar results were obtained at pH 7, though here the differences in the total O_2 uptakes

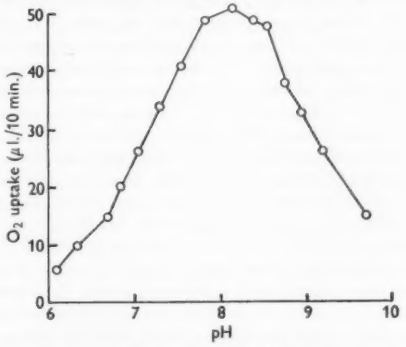

Fig. 1. Effect of pH on the rate of oxidation of tryptamine. Amine oxidase (0·1 ml.) and catalase in phosphate–borate buffers. The final concentration both of phosphate and borate was 0·033M. The substrate, 0·2 ml. of 0·1M tryptamine, was added from the side arms; KOH was omitted from the centre wells. The total volume of the reaction mixtures was 3 ml. Gas phase, air; temp., 28°. Control reaction mixtures without tryptamine showed no oxygen uptake in 10 min.

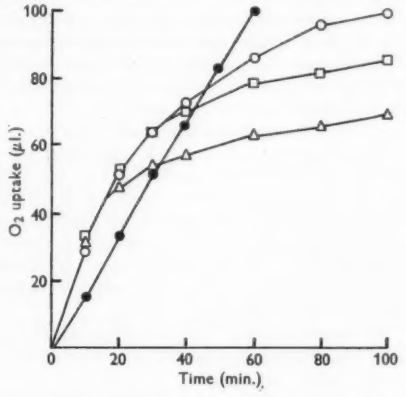

Fig. 2. Enzyme inactivation or inhibition produced by high tryptamine concentrations. Amine oxidase (0·08 ml.) and catalase in 0·033M phosphate buffer, pH 8, with varying concentrations of tryptamine; KOH was present in the centre wells. Other conditions were similar to those in Fig. 1. ○, 0·0033M Tryptamine; □, 0·0067M tryptamine; △, 0·02M tryptamine; ●, 0·02M 2-phenylethylamine (0·02 ml. of amine oxidase).

were smaller. This was due, at least in part, to the fact that the initial reaction rates at this pH were greater at the higher substrate concentrations.

Werle & Pechmann (1949) showed that plant amine oxidase is inactivated during the course of the reactions it catalyses, particularly at high substrate concentrations, and attributed the inactivation to the hydrogen peroxide formed as a reaction product. Mann (1955) showed that the enzyme is readily inactivated by hydrogen peroxide only in presence of its substrates, and that with 1:4-diaminobutane or 1:5-diaminopentane (0·01 M) as substrate the inactivation is prevented by catalase. The amounts of catalase used in the present work were sufficient to prevent inactivation of the enzyme when 2-phenylethylamine (0·02 M) was used as substrate (Fig. 2). The decreasing O_2 uptakes with increasing tryptamine concentrations were unaffected by a tenfold increase in catalase. These results show that the decreasing O_2 uptake is dependent on the fact that tryptamine was used as substrate. In experiments with animal diamine oxidase Zeller, Schär & Staehlin (1939) and Zeller (1941) showed that substrates such as histamine and agmatine, which contain two basic groups with very different affinities for the enzyme, act as inhibitors at high concentrations. Such inhibition, like that observed by Mann & Smithies (1955) using plant amine oxidase with 2-(2-aminophenyl)ethylamine as substrate, and unlike that with tryptamine, is apparent from the start of the reaction. Further work is necessary to establish whether inactivation by hydrogen peroxide or by excess of substrate is the cause of the decreasing O_2 uptakes. A third possibility is that combination takes place between tryptamine and its oxidation product to form an inhibiting compound; this would be favoured by high tryptamine concentrations.

Oxidation of 5-hydroxytryptamine. The oxidation of 5-hydroxytryptamine is also catalysed by the enzyme. In phosphate buffer, pH 7, the initial rate of O_2 uptake with 5-hydroxytryptamine creatinine sulphate (10 μmoles) as substrate was about one-half of that with the same concentration of tryptamine. No O_2 uptake was observed in control experiments in which creatinine sulphate was tested as substrate.

Total oxygen uptake. The reactions catalysed by amine oxidases have generally been represented by the equation

$$R.CH_2.NH_2 + H_2O + O_2 = R.CHO + H_2O_2 + NH_3.$$

In presence of catalase the net reaction is expressed

$$2R.CH_2.NH_2 + O_2 = 2R.CHO + 2NH_3.$$

If the reaction studied consists only of the oxidation of tryptamine to IAc, the total O_2 uptake in the presence of catalase should be 0·5 mole of O_2/mole of tryptamine. The total O_2 uptake was measured, in the presence of catalase, in phosphate buffers, pH 7 and 8. Since the amine oxidase is inhibited or inactivated during the reaction sufficient enzyme was used to give a rapid initial rate of O_2 uptake, and low concentrations of tryptamine were used. The results obtained at pH 7 are shown in Fig. 3; those at pH 8 were similar. With the purified enzyme as catalyst the O_2 uptake rapidly reached a value slightly greater than 0·5 mole/mole of tryptamine, but thereafter further uptake took place though this was relatively slow. The total O_2 uptake reached in the experimental time was about 0·6 mole. No CO_2 was formed in the reaction. The results suggest that the oxidation of tryptamine to IAc is the main reaction with the purified enzyme as catalyst. The excess of O_2 uptake over the theoretical with the purified enzyme was not inhibited by pyrophosphate and was not therefore the same as that observed by Mann (1955), using lysine as substrate.

When the dry preparations of amine oxidase were used to catalyse the reaction the O_2 uptake reached

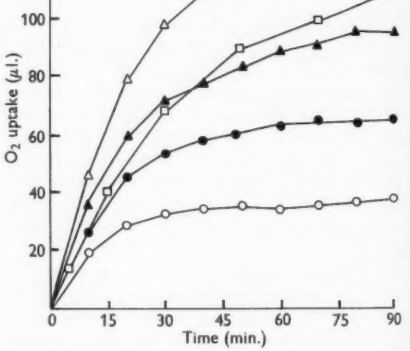

Fig. 3. Course of the oxygen uptake during the catalysis of the oxidation of tryptamine by the purified and dry preparations of amine oxidase. Amine oxidase (0·2 ml. of the purified preparation or 100 mg. of the dry preparation) and catalase in 0·033 M phosphate buffer, pH 7; KOH was present in the centre wells. Other conditions were similar to those in Fig. 2. The oxygen uptakes of control reaction mixtures without tryptamine, 0–5 μl. with the purified amine oxidase and 52 μl. with the dry preparation, were subtracted. ○, 2·5 μmoles of tryptamine; ●, 5 μmoles of tryptamine; ▲, 7·5 μmoles of tryptamine; △, 10 μmoles of tryptamine with the purified amine oxidase; □, 5 μmoles of tryptamine with the dry preparation of amine oxidase. Oxygen uptakes of 0·5 mole equivalents would be 28, 56, 84 and 112 μl. with 2·5, 5, 7·5 and 10 μmoles of tryptamine.

1 mole/mole of tryptamine (Fig. 3) and exceeded this value when the experimental time was increased. This higher O_2 uptake with the dry preparation may have been due to the oxidation of IAc, possibly to IAA. When the purified enzyme was used to catalyse the oxidation of tryptamine the final reaction mixtures gave a golden-yellow colour with Salkowski reagent, but when the dry preparation was used the reaction mixtures, like IAA, gave a red colour with the reagent. The yield of IAA, assuming the optical density at 530 mμ. to be entirely due to this compound, was very small. Thus in the experiment of Fig. 3 where 5 μmoles of tryptamine were oxidized with the dry preparation the IAA estimated to be present was less than 0·04 μmole.

Oxidation of phenylacetaldehyde and IAA by the dry preparation. No significant increase in O_2 uptake was found when acetaldehyde, benzaldehyde, salicylaldehyde and hypoxanthine were tested as substrates for the dry preparation. The dry preparation, but not the purified preparation, catalysed the oxidation of phenylacetaldehyde and IAA (Table 1). Kenten (1953) has shown that the oxidation of phenylacetaldehyde is catalysed by many plant saps and that in pea-seedling sap the oxidizing system consists of a peroxidase together with a thermostable factor which can be partially replaced by Mn^{2+} ions. The oxidation of IAA, catalysed by horse-radish peroxidase, is also stimulated by Mn^{2+} ions (Kenten, 1955*a*). The dry preparation of amine oxidase had P.Z. 1 and contained 12 μg. of manganese/100 mg. The peroxidase activity of the amounts of purified amine oxidase used (0·2 ml.) was only about 1–2 % of that of 100 mg. of the dry preparation. Amounts of peroxidase and Mn^{2+} of the same order as those present in 100 mg. of the dry preparation catalysed the oxidation of phenylacetaldehyde and IAA (Table 1).

Oxidation of the product of the amine oxidase reaction by the dry preparation and by peroxidase. When the oxidation of tryptamine, catalysed by the purified amine oxidase, was allowed to proceed until the O_2 uptake reached 0·5 mole equivalent little increase was produced in the subsequent slow rate of O_2 uptake by adding more of the purified amine oxidase. If the dry preparation was added at this point, instead of the purified preparation, the rate of oxidation was markedly increased and the total O_2 uptake approached 1 mole equivalent (Fig. 4). This further oxidation was also catalysed by peroxidase, particularly in presence of Mn^{2+} ions. This was shown in similar experiments in which, after the initial oxidation was complete, peroxidase, Mn^{2+} ions or peroxidase together with Mn^{2+} ions were added from the second side arm (Fig. 5). Peroxidase caused an increased rate of O_2 uptake which differed little whether 0·1 or 0·3 mg. of peroxidase was used. The Mn^{2+} ions (11 μg.) alone had no effect on O_2 uptake but added together with peroxidase produced a larger effect than that given by peroxidase alone. Heat-treated peroxidase (30 min. at 100°), with or without Mn^{2+} ions, only slightly increased the rate of O_2 uptake. The results suggest that the greater O_2 uptake observed when the dry preparation of amine oxidase was used instead of the purified preparation, to catalyse the oxidation of tryptamine, was due at least in part to the oxidation of the product of the amine oxidase reaction by a peroxidase system. Results reported below show that the product of the amine oxidase reaction is IAc. Tests with Salkowski reagent showed that, in the experiments of Fig. 5, only traces of IAA accumulated in the reaction mixtures whether peroxidase or peroxidase together with Mn^{2+} ions was used to catalyse the secondary oxidation.

Table 1. *Oxidation of phenylacetaldehyde and IAA by the dry preparation of amine oxidase and by peroxidase ± Mn^{2+} ions*

Purified or dry preparations of amine oxidase, or peroxidase, with and without $MnSO_4$, were tested as catalysts for the oxidation of phenylacetaldehyde or IAA in reaction mixtures containing catalase in 0·033 M phosphate buffer, pH 7. The substrates, 10 μmoles of phenylacetaldehyde or IAA, were added from the side arms. In the experiments with phenylacetaldehyde KOH was omitted from the centre wells and measurements of the O_2 uptake were not started until 5 min. after the addition of the substrate, which was dissolved in ethanol (0·1 ml.). The O_2 uptakes of control reaction mixtures of the dry preparation of amine oxidase without substrate were subtracted. These were 10 μl. (KOH absent) and 28 μl. (KOH present). Other conditions were similar to those in Fig. 2.

Catalyst	Substrate	O_2 uptake (μl./1 hr.)
Amine oxidase (100 mg. of dry preparation)	Phenylacetaldehyde	57
Amine oxidase (100 mg. of dry preparation)	IAA	53
Purified amine oxidase (0·2 ml.)	Phenylacetaldehyde	0
Purified amine oxidase (0·2 ml.)	IAA	5
Peroxidase (0·1 mg.)	Phenylacetaldehyde	22
Mn^{2+} ions (11 μg.)	Phenylacetaldehyde	8
Peroxidase (0·1 mg.) + Mn^{2+} ions (11 μg.)	Phenylacetaldehyde	44
Peroxidase (0·1 mg.)	IAA	48
Mn^{2+} ions (11 μg.)	IAA	0
Peroxidase (0·1 mg.) + Mn^{2+} ions (11 μg.)	IAA	63

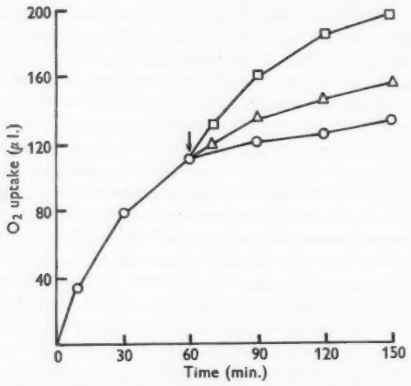

Fig. 4. Oxidation of the product of the amine oxidase-catalysed reaction by the dry crude preparation of amine oxidase. Amine oxidase (0·2 ml. of the purified preparation) and catalase in 0·033 M phosphate buffer, pH 7, in reaction vessels with two side arms. Tryptamine (10 µmoles) was added from the side arm. Other conditions were similar to those in Fig. 1. ○, No further addition; △, an additional 0·2 ml. of purified amine oxidase was added from the second side arm at ↓; □, 60 mg. of the dry preparation of amine oxidase suspended in 0·3 ml. of water was added at ↓. The oxygen uptakes of control reaction mixtures without added substrate were subtracted. These were 4 µl. with 0·2 ml. of amine oxidase, increasing to 6 µl. where a further 0·2 ml. was added and to 46 µl. where the second addition was that of the dry preparation.

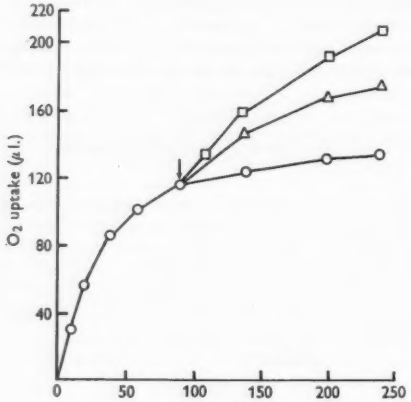

Fig. 5. Oxidation of the product of the amine oxidase-catalysed reaction by peroxidase ± Mn^{2+} ions. Conditions were as in Fig. 4 but the additions from the second side arms at ↓ were: ○, no addition or 0·2 ml. of mM-$MnSO_4$ (11 µg. of Mn^{2+} ions); △, 0·1 mg. of peroxidase; □, 0·1 mg. of peroxidase + 11 µg. of Mn^{2+} ions.

Oxidation of tryptamine by peroxidase systems. Another secondary reaction which may occur when the dry preparation is used as catalyst is the oxidation of tryptamine by the hydrogen peroxide formed in the primary reaction. Colorimetric evidence suggesting that peroxidase catalyses this oxidation was obtained in test-tube experiments at room temperature. Reaction mixtures of a total volume of 3 ml. contained 1 mg. of peroxidase and 20 µmoles of tryptamine in 0·067 M phosphate buffer, pH 7. Additions of 0·05 ml. of 0·02 M hydrogen peroxide were made at intervals of 10 min. over a period of 200 min. The reaction mixtures turned yellow after the first addition of hydrogen peroxide. This colour slowly deepened with subsequent additions of hydrogen peroxide and after 200 min. an orange-brown solution with slight turbidity resulted. After standing at room temperature for a further period of several hours a brown precipitate was deposited. No colour or precipitate formation was observed in reaction mixtures from which peroxidase was omitted or in which heat-treated peroxidase (30 min. at 100°) was used. As in the peroxidase-catalysed oxidation of tryptophan (Wiltshire, 1953) the reaction apparently depends on the maintenance of a low concentration of hydrogen peroxide. When the hydrogen peroxide (1 ml. of 0·02 M) was all added at the start of the reaction the subsequent visual signs of tryptamine oxidation were comparatively slight.

The oxidation of tryptamine by hydrogen peroxide in the presence of peroxidase was also demonstrated in manometric experiments in which the notatin-catalysed oxidation of glucose was used as the source of the hydrogen peroxide. The basal reaction mixture consisted of notatin, catalase and glucose (10 µmoles) in phosphate buffer, pH 7. Tryptamine (10 µmoles) or peroxidase (0·1 or 1 mg.) added separately to such reaction mixtures had no effect on the O_2 uptake, but where both were added together the O_2 uptake was increased and was doubled with the larger amount of peroxidase (Table 2). These increases in O_2 uptake were accompanied by the formation of an orange-brown colour in the reaction mixtures where 0·1 mg. of peroxidase was used and a brown precipitate with 1 mg. of peroxidase. Heat treatment of the peroxidase (30 min. at 100°) decreased, but did not entirely eliminate, the peroxidase effect. The effect of peroxidase (0·1 mg.) was not increased by the addition of Mn^{2+} ions (1·1 or 11 µg.).

Identification of IAc as the oxidation product of tryptamine

Spectrophotometric evidence. When the oxidation of tryptamine, catalysed by amine oxidase in the presence of catalase, was allowed to proceed until the O_2 uptake reached 0·5 mole equivalent, samples of the reaction mixture gave a golden-yellow

Table 2. *Oxidation of tryptamine by peroxidase systems*

The basal reaction mixture consisted of tryptamine (10 μmoles), notatin (0·15 mg.) and catalase in 3 ml. of 0·033 M phosphate buffer, pH 7. Glucose (10 μmoles) was added from the side arms. Other reactions contained, in addition, peroxidase, $MnSO_4$ or heat-treated peroxidase (30 min. at 100°); KOH was present in the centre wells. Other conditions were as in Fig. 2.

Catalyst	O_2 uptake (μl./4 hr.)
Notatin	108
Notatin + 11 μg. of Mn^{2+} ions	106
Notatin + 0·2 mg. of peroxidase	168
Notatin + 0·2 mg. of peroxidase + 1·1 μg. of Mn^{2+} ions	165
Notatin + 0·2 mg. of peroxidase + 11 μg. of Mn^{2+} ions	158
Notatin + 1 mg. of peroxidase	228
Notatin + 0·2 mg. of peroxidase (heat-treated)	115
Notatin + 1 mg. of peroxidase (heat-treated)	134

Table 3. *Spectrophotometric evidence of the formation of IAc as the product of the oxidation of tryptamine catalysed by amine oxidase*

Reaction mixtures consisted of amine oxidase (0·2 ml.), catalase and tryptamine (10 μmoles) in 3 ml. of 0·033 M phosphate buffer, pH 7. Other conditions were as in Fig. 2. The reaction mixtures were incubated for 1 hr., at which time the O_2 uptake had reached 0·5 mole equivalent. Control reaction mixtures, without tryptamine, were incubated for the same time. After addition of Salkowski reagent to samples of the reaction mixture, before and after various treatments, the optical density at 530 mμ. was measured. In each case 0·25 ml. of the reaction mixture, or equivalent amounts of the treated reaction mixtures, was diluted to 10 ml. with water and 15 ml. of Salkowski reagent was added. The blank cells contained Salkowski reagent and control reaction mixtures without tryptamine treated in the same way as the experimental mixture.

Sample	Treatment	Colour with Salkowski reagent	Optical density at 530 mμ.
Reaction mixture (0·25 ml.)	None	Golden-yellow	0·053
Reaction mixture (0·25 ml.)	0·125 ml. of a sat. soln. of 2:4-dinitrophenylhydrazine in 2 N-HCl added. Bulky orange-brown ppt. removed by centrifuging	Yellow*	0·006
Reaction mixture (0·25 ml.)	0·125 ml. of 2 N-HCl added. Slight ppt. removed by centrifuging	Golden-yellow	0·047
Reaction mixture (0·25 ml.)	Oxidized with silver oxide	Red	0·258
Reaction mixture (0·25 ml.)	Oxidized with silver oxide, then treated with 2:4-dinitrophenyhydrazine reagent. No ppt. formed	Red	0·260
Tryptamine (0·83 μmole)	None	None	0·007
Tryptamine (0·83 μmole)	Oxidized with silver oxide	None	0·008
IAA (0·83 μmole)	None	Red	0·362

* This colour was due, at least in part, to the presence of 2:4-dinitrophenylhydrazine.

solution on treatment with Salkowski reagent. The optical density of these coloured solutions at 530 mμ., where IAA treated with Salkowski reagent shows an absorption maximum, was low in relation to that required for quantitative conversion of the tryptamine into IAA (Table 3). When the reaction mixture was treated with 2:4-dinitrophenylhydrazine in 2 N-HCl a bulky orange-brown precipitate was formed. If this precipitate was removed at once by centrifuging, the supernatant treated with Salkowski reagent showed no absorption at 530 mμ. Treatment of the reaction mixture in the same way with 2 N-HCl alone produced little or no precipitate and the optical density at 530 mμ. remained almost unchanged. These results suggested that the observed absorption was due to the presence of a carbonyl compound. When the reaction mixture was oxidized with silver oxide, under the conditions described for estimation of IAc, the resultant solution, like IAA, gave a red colour with Salkowski reagent; the optical density at 530 mμ. suggested that about 70% of the tryptamine was recovered as IAA. Tryptamine gave little or no colour with Salkowski reagent before or after silver oxide treatment. The reaction mixture oxidized with silver oxide gave no precipitate with 2:4-dinitrophenylhydrazine, nor was the optical density at 530 mμ. affected by this reagent. The results suggested that IAA was formed by the oxidation of the carbonyl compound in the reaction

mixture. This is supported by the absorption curve of the silver oxide-treated mixture after the addition of the Salkowski reagent and 2:4-dinitrophenylhydrazine. Fig. 6 shows that the absorption curve was almost identical with that of IAA. A red colour and a very similar absorption curve were obtained with the reaction mixture oxidized by silver oxide with the more specific Salkowski reagent described by Gordon & Weber (1951), in which perchloric acid is substituted for sulphuric acid. Stowe & Thimann (1954) have shown that the coloured products this modified reagent forms with compounds related to IAA have different absorption maxima. Since pretreatment with 2:4-dinitrophenylhydrazine abolishes the observed absorption of the reaction mixture at 530 mμ. but does not affect it after oxidation with silver oxide the absorption after the latter treatment may be taken as a measure of the IAA present, and from this the amount of IAc in the original reaction mixture can be calculated.

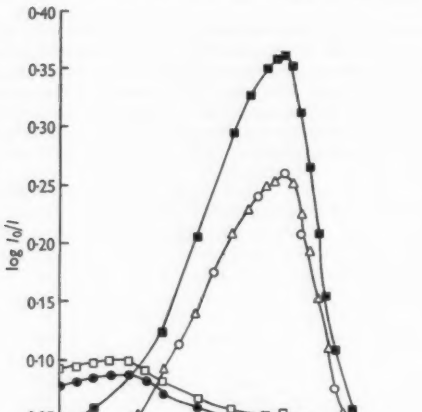

Fig. 6. Absorption spectra of the solutions obtained by mixing samples of the reaction mixture, before and after various treatments, with Salkowski reagent. Reaction mixtures containing 0·2 ml. of amine oxidase, catalase and tryptamine (10 μmoles) were incubated in 0·067 M pyrophosphate buffer, pH 7, until the oxygen uptake reached 0·5 mole equivalent. Control reaction mixtures without tryptamine were incubated for the same time. Other conditions were as in Fig. 2. In the Salkowski reaction 0·25 ml. of the untreated reaction mixture, or an equivalent amount of the treated reaction mixtures, was used. The blank cell contained appropriate controls made by mixing Salkowski reagent with samples of the control reaction mixture similarly treated. □, Untreated reaction mixture; ▲, supernatant after removal of 2:4-dinitrophenylhydrazone; ●, supernatant after treatment with 2 N-HCl; △, reaction mixture treated with silver oxide; ○, supernatant after oxidation with silver oxide and treatment with 2:4-dinitrophenylhydrazine; ■, 0·83 μ-mole of IAA.

Paper chromatography. Reaction mixtures, of a total volume of 3 ml., contained 0·3 ml. of amine oxidase and catalase in 0·002 M phosphate buffer, pH 7; KOH was omitted from the centre wells. Tryptamine (10 μmoles) was added from the side arms. The reaction mixtures were incubated until O_2 uptake was about 0·5 mole equivalent. One of the reaction mixtures was then shaken in the bath for a further 10 min. after the addition of 0·5 ml. of a suspension of silver oxide. The silver oxide was prepared immediately before use by mixing 10 ml. of 0·1 M-$AgNO_3$ with 10 ml. of 0·1 M-NaOH. The precipitated silver oxide was collected and washed by centrifuging and suspended in 2 ml. of water. Finally both reaction mixtures were filtered and the filtrates were concentrated in a vacuum desiccator until the volume of each was about 0·3 ml. Samples (0·005 ml.) were then chromatographed with the *iso*propanol solvent, with Ehrlich reagent as detecting agent. Tryptamine and IAA were used as reference compounds. The reaction mixture not treated with silver oxide gave a strong yellow-brown streak (R_F 0·90) and a weak purple spot identical with that given by tryptamine (R_F 0·73). Neither of these spots was given by control reaction mixtures without tryptamine nor by the reaction mixtures treated with silver oxide, but a new purple spot appeared with the latter identical with that given by IAA (R_F 0·44). Control reaction mixtures of tryptamine and heat-treated enzyme (10 min. at 100°) both before and after treatment with silver oxide showed only the one spot corresponding to tryptamine. The results suggest that the yellow-brown streak (R_F 0·90) was due to IAc, or to an artifact formed during the chromatography, and that the IAc was oxidized by the silver oxide to IAA. Weller, Wittwer & Sell (1954) record that IAc gives a yellow-brown colour with Ehrlich reagent on paper chromatograms.

Conditions affecting the yield of IAc. The effects of pH, reaction time and tryptamine concentration on the yield of IAc were investigated. The reaction mixture, in phosphate buffers at pH 7 and 8, were incubated until the O_2 uptakes reached 0·5 mole equivalent and the ammonia and IAc present were then estimated. The results (Table 4) show that the yields of IAc were of the order of 90 % of theoretical with the lowest concentration of tryptamine used but decreased progressively as the tryptamine concentration was increased. The decrease was due, at least in part, to the longer reaction times. Thus in the experiments with 30 μmoles of tryptamine the

Table 4. *Effect of tryptamine concentration*, pH *and oxidation time on the yield of IAc*

Reaction mixtures of 9 ml. total volume in 60 ml. Warburg vessels consisted of amine oxidase and catalase in 0·033 M phosphate buffers, pH 7 and 8. The substrate was added from the side arms; KOH was omitted from the centre wells. When the O_2 uptakes reached 0·5 mole equivalent the ammonia and IAc contents of the reaction mixtures were estimated. Values obtained with control reaction mixtures without tryptamine were subtracted.

Tryptamine (μmoles)	pH	Amine oxidase (ml.)	Time of incubation (min.)	Total O_2 uptake (μmoles)	Ammonia (μmoles)	IAc (μmoles)
15	7	0·6	15	7·5	15·3	13·0
15	8	0·3	10	7·8	15·2	13·9
30	7	0·6	25	14·8	27·3	24·3
30	8	0·3	15	15·0	27·6	23·2
30	7	0·3	70	15·2	25·2	19·8
30	8	0·15	60	15·2	27·3	17·7
60	7	0·6	80	30·6	48·0	40·7
60	8	0·3	140	29·4	45·6	29·1

yields of IAc fell when the reaction times were increased by using less of the enzyme preparation. The decrease in yield at the higher tryptamine concentrations was accompanied by the formation of an insoluble reaction product. The amount of this was greater at pH 8 than at pH 7 and was probably due, in part, to polymerization of IAc. Kenten (1953) found that aqueous solutions of phenylacetaldehyde rapidly became turbid in alkaline solution, presumably owing to polymerization. But the fact that, in the tryptamine reaction, the yield of ammonia also decreased with increase in tryptamine concentration, shows that reactions other than polymerization were involved. Some pigment formation occurred during the reaction, ranging from a slight yellow at low tryptamine concentrations to yellow-brown at high concentrations. It was more marked at pH 7 than at pH 8, and was apparently due to a highly dispersed insoluble product, since the supernatant solution obtained after centrifuging was almost colourless.

Isolation of the 2:4-*dinitrophenylhydrazone of IAc.* Reaction mixtures in 250 ml. Erlenmeyer flasks each consisted of 1·6 ml. of amine oxidase, 0·6 mg. of catalase, 1·2 ml. of 0·1 M tryptamine and 6 ml. of 0·2 M phosphate buffer, pH 7, in a total volume of 36 ml. Five such reaction mixtures were used. The flasks were shaken in the bath at 28° for 75 min. A previous control experiment had shown that the O_2 uptake reached 0·5 mole equivalent under these conditions. The combined reaction mixtures and washings were brought to pH 4–5 with conc. HCl and centrifuged. The supernatant was added to 50 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl. After the addition of 50 ml. of 2 N-HCl the mixture was stored at 4° overnight. The orange-yellow precipitate (165 mg., 82 % of theoretical yield), m.p. 184–188°, which formed, was separated by filtration and dried *in vacuo*. Part of the product (49 mg.) was gently warmed with benzene (5 ml.), filtered free from insoluble material (7 mg.) and the solution chromatographed on a kieselguhr–bentonite (5 g.:5 g.) column (Linstead, Elvidge & Whalley, 1955) by elution with benzene. The eluate was collected in 8 ml. portions. Evaporation of the combined fractions 3–6 and crystallization from a supersaturated benzene solution gave small orange-red prisms, m.p. 196–198°. (Found: C, 57·0; H, 3·9; N, 20·6. Calc. for $C_{16}H_{13}O_4N_5$: C, 56·6; H, 3·85; N, 20·65 %.) Light-absorption max., 2690 and 3590 Å (ε 14 700 and 22 000 respectively). Brown, Henbest & Jones (1952) give m.p. 196–202°, light-absorption max., 2680 and 3580 Å (ε 16 800 and 23 600 respectively).

Isolation of the dimedone derivative of IAc. A total of 6 ml. of 0·1 M tryptamine was oxidized in reaction mixtures similar to those used for the preparation of the 2:4-dinitrophenylhydrazone. The combined reaction mixtures were brought to pH 5 with conc. HCl and extracted three times with ether, a total of 300 ml. of ether being used. The combined ether extracts were dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure left a viscous oil which was immediately dissolved in 10 ml. of aqueous methanol (1:1) and treated with a solution of dimedone (300 mg.) in the same solvent. The mixture after storage at 4° overnight had deposited 72 mg. of crystals. After storing the filtrate for 24 hr. at 4° a further 18 mg. was obtained. The total yield (90 mg.) was 36 % of theoretical. Recrystallization from aqueous methanol (1:1) gave colourless crystals, m.p. 150–151°. Admixture with dimedone gave m.p. 122–127°. (Found: C, 73·7; H, 7·6. Calc. for $C_{25}H_{31}O_4N$: C, 74·1; H, 7·45 %.) Brown *et al.* (1952) give m.p. 148–152°.

DISCUSSION

The results of the present work establish the fact that IAc is the product of the oxidation of tryptamine catalysed by plant amine oxidase. This is of interest in connexion with the metabolism of indolylalkylamines in both animals and plants.

In experiments with intact animals and perfused organs it has been shown that tryptamine is oxidized to IAA (Ewins & Laidlaw, 1913; Guggenheim & Loeffler, 1916) and that 5-hydroxytryptamine is oxidized to 5-hydroxy-IAA (Titus & Udenfriend, 1954; Erspamer, 1954, 1955). It is known that the oxidation of tryptamine (Blaschko, Richter & Schlossmann, 1937; Pugh & Quastel, 1937) and of 5-hydroxytryptamine (Blaschko & Philpot, 1953; Govier, Howes & Gibbons, 1953) is catalysed by the monoamine oxidase of animal tissues. This reaction is considered to be the first stage in the oxidation of these amines in animals, but no conclusive proof appears to have been obtained hitherto that, with these two amines as substrates, the corresponding aldehydes are the reaction products. The importance of these observations in animal metabolism depends on the pharmacological properties of certain naturally occurring indolylalkylamines, notably 5-hydroxytryptamine and its *N*-methyl derivatives. 5-Hydroxytryptamine occurs in animal tissues, and its oxidation product 5-hydroxy-IAA is a normal constituent of the urine (Erspamer, 1955; Udenfriend, Titus & Weissbach, 1955). 5-Hydroxytryptamine is formed from tryptophan by way of 5-hydroxytryptophan (Udenfriend, Titus, Weissbach & Peterson, 1956), which is then decarboxylated by the specific 5-hydroxytryptophan decarboxylase described by Udenfriend, Clark & Titus (1953) and Clark, Weissbach & Udenfriend (1954). The presence of IAA in urine was established by Herter (1908). Both IAA and indoleaceturic acid have since been found repeatedly as normal constituents of urine. But it is not yet clear whether such IAA is, in part, a product of metabolism of animal tissues or whether it is derived entirely from plant material in the diet and bacterial action on tryptophan in the intestine.

The literature on the mechanism of formation of IAA in plant tissues has been reviewed recently by Larsen (1951*a*) and Gordon (1954, 1956). Insufficient evidence is at present available to permit an assessment of the importance of the reaction studied in the present work in this connexion. The presence of tryptamine in certain *Acacia* species was established by White (1944) but there is no evidence, as yet, that it is widely distributed in plants. Isolated instances of the occurrence in plants of 5-hydroxytryptamine (Bowden, Brown & Batty, 1954), *NN*-dimethyltryptamine (Fish, Johnson & Horning, 1955) and 5-hydroxy-*NN*-dimethyltryptamine (Stromberg, 1954; Fish *et al.* 1955) have been reported. These findings suggest that the corresponding substituted indoleacetic acids may also be present in plants. Some plants are apparently unable to utilize tryptamine for the formation of IAA. Gordon (1956) reports that inhibitors of amine oxidase did not affect the formation of IAA from tryptophan by a plant-tissue preparation and suggests that tryptamine is not a normal intermediate in auxin formation. Evidence that IAc occurs in some higher plants and can act as a precursor of IAA has been obtained by Larsen (1944, 1949, 1951*b*), Gordon & Sanchez-Nieva (1949*a*, *b*) and by Yamaki & Nakamura (1952).

The extent to which amine oxidase-catalysed oxidation of tryptamine occurs in plants may be restricted not only by the distribution of tryptamine but by that of the amine oxidases. Plant amine oxidase is known to be present in many leguminous plants. In the most complete survey of its distribution so far undertaken, based on its histaminase activity, Werle & Zabel (1948) obtained evidence of its presence in many other dicotyledonous plants but it was not found in the monocotyledons and gymnosperms examined. Little is known of the distribution of the plant monoamine oxidase which was shown to catalyse the oxidation of tryptamine by Werle & Roewer (1950).

It is now clear that the failure to identify IAc as the product of the oxidation of tryptamine in previous experiments with crude preparations of plant amine oxidase was due to the secondary reactions catalysed by such preparations. The results suggest that two of these reactions are peroxidase-catalysed oxidations of tryptamine and of IAc. Such oxidation of tryptamine was found to depend on an external source of hydrogen peroxide and was unaffected by Mn^{2+} ions at the concentrations used. The oxidation of IAc was independent of an external source of hydrogen peroxide and the rate of oxidation was increased by Mn^{2+} ions. Kenten (1953) identified benzaldehyde as the main product of the oxidation of phenylacetaldehyde by peroxidase systems and suggested that if IAc was oxidized by such systems 3-indolealdehyde would be a possible reaction product. In the present work tests with Salkowski reagent suggest that only traces of IAA accumulate during the reaction though this does not exclude the possibility that IAA is an intermediate since peroxidase systems also catalyse the oxidation of IAA (e.g. Galston, Bonner & Baker, 1953; Kenten, 1955*a*).

It is already known that the oxidation of indole (Mann & Smithies, 1955) and that of a number of its compounds is catalysed by peroxidase systems. Besides IAA these compounds include tryptophan (Wiltshire, 1953), β-(3-indolyl)propionic acid and γ-(3-indolyl)-*n*-butyric acid (Kenten, 1955*b*). An oxidation of tryptamine to give 5-hydroxytryptamine, a reaction already discussed by Erspamer & Vialli (1952), may also occur. In the present work preliminary colorimetric tests for 5-hydroxytryptamine as a product of the oxidation of tryptamine by peroxidase systems with 1-nitroso-2-naphthol (Udenfriend, Weissbach & Clark, 1955) have so far

given negative results. The pigment formation occurring during the oxidation suggests that the final products are complex. Pigment formation was also observed during the oxidation of tryptamine (Pugh & Quastel, 1937) and of 5-hydroxytryptamine (Blaschko, 1952) catalysed by preparations of the monoamine oxidase of animal tissues. This pigment formation has been further studied by Blaschko & Hellmann (1953), who attribute it to the products of the oxidation of the aldehydes formed in the oxidative deamination reactions.

Lastly it should be pointed out that the amine oxidase-catalysed oxidation of tryptamine provides a simple means of obtaining IAc. The possibility that this might prove to be the case was suggested by Gordon (1954). The work should therefore facilitate investigation designed to assess the importance of IAc as a precursor of IAA in plants. Much of the previous work on this subject has been done with preparations of IAc made by treating tryptophan with ninhydrin or isatin and containing only 2% of IAc, or by the use of plant extracts containing IAc. While a chemical method of synthesis is now available by which pure IAc can be obtained (Brown *et al.* 1952), because of the instability of the compound the enzymic method of preparation has obvious advantages for the proposed type of work. It may also be possible to prepare 5-hydroxy-IAc by the oxidation of 5-hydroxytryptamine, catalysed by the amine oxidase.

SUMMARY

1. Plant amine oxidase catalyses the oxidation of tryptamine to 3-indolylacetaldehyde (IAc). This was established by the isolation of the 2:4-dinitrophenylhydrazone and dimedone derivatives of IAc from the reaction mixtures.

2. The oxidation of 5-hydroxytryptamine is also catalysed by the enzyme.

3. A method has been worked out for the estimation of IAc based on its oxidation, with silver oxide, to 3-indolylacetic acid (IAA). The IAA so formed was estimated with Salkowski reagent.

4. With this method the effect of pH, substrate concentration and reaction time on the yield of IAc was investigated. The enzyme was rapidly inactivated at high tryptamine concentrations. With low tryptamine concentrations and short reaction times (15 min.) yields of IAc of up to 90% of theoretical were obtained at pH 7–8. The yield decreased with increase in reaction time, particularly with alkaline reaction mixtures. This was probably due, in part at least, to polymerization of the IAc.

5. It is suggested that the reaction forms a useful method of obtaining IAc.

6. Two of the secondary reactions which occur when crude preparations of the amine oxidase are used to catalyse the reaction were identified as peroxidase-catalysed oxidations of tryptamine and IAc.

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The Excretion of Bilirubin as a Diglucuronide Giving the Direct van den Bergh Reaction

By BARBARA H. BILLING,* P. G. COLE and G. H. LATHE
The Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W. 6, and the Institute of Obstetrics and Gynaecology, British Postgraduate Medical Federation, University of London

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van den Bergh & Müller (1916) reported that there was a difference between bilirubin and the bilirubin-like pigment which is excreted in bile. They observed that bilirubin, and the serum pigment of patients with haemolytic jaundice, required ethanol for coupling with diazotized sulphanilic acid in acid solution. These materials were said to give an 'indirect' reaction. In contrast, the pigment of bile, and of sera from patients with obstructive jaundice and hepatitis, reacted 'directly', without the addition of ethanol. Cole & Lathe (1953) found that the direct and indirect types of pigment could be separated chromatographically, and Cole, Lathe & Billing (1954) showed that the direct-reacting pigment had two components (pigments I and II). Pigment II is the more polar of the two, and is also the chief pigment of human bile. It occurs, with pigment I, in the serum and urine of patients with obstructive jaundice and hepatitis.

Billing (1954) compared the products of diazotization of bilirubin and of pigment II and found that the azo pigment (pigment B) formed from pigment II was more polar than pigment A formed from bilirubin. The relation between these pigments is shown in Fig. 1. It was suggested that the difference between bilirubin and pigment II could

* Present address: Royal Veterinary College, London, N.W. 1.

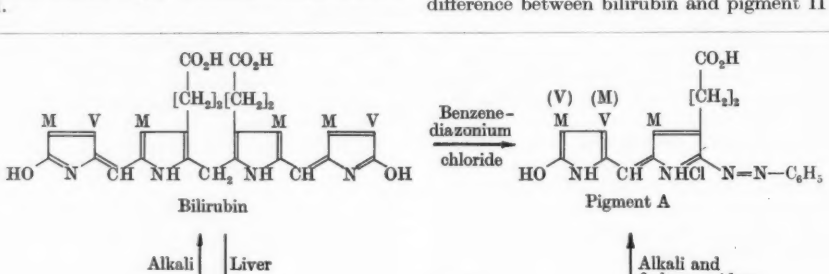

Fig. 1. Relation between bilirubin, pigment II and azo pigments A and B. M, Methyl; V, vinyl.

be predicted from a knowledge of the difference in structure of pigments A and B. The structure of pigment A was established by Fischer & Haberland (1935). Pigment B has now been isolated and shown to be a glucuronide of pigment A. A preliminary report of this work has already appeared (Billing & Lathe, 1956).

Since bilirubin is asymmetrical (Siedel & Fischer, 1933), pigments A and B must each consist of a mixture of two isomers, differing only in the position of the methyl and vinyl side chains. There is some evidence that the isomers have slightly different R_F values on partition chromatograms, but they behave very similarly, and in this work the two isomers of each pigment are discussed as if they were one substance.

EXPERIMENTAL

Analytical

Pigments. Diazotizable bile pigments were estimated by the method of Malloy & Evelyn (1937). Pigments A and B were determined, after dilution in methanol, with a methyl red standard (King, 1951) in a Unicam colorimeter (SP. 300) and an Ilford filter no. 404 (peak transmission 530 mμ.). The standard was equivalent to 4 μg. of bilirubin/ml. and equivalent to 5·12 μg. of pigment A (the benzenediazonium chloride derivative)/ml. At the beginning of the study the nature of pigment B was unknown and it was necessary to express its concentration in terms of pigment A.

Glucuronic acid. Qualitative tests for hexuronic acids were made by the carbazole–H_2SO_4 method of Dische (1947*a*). Glucuronic acid was identified by the mannose–H_2SO_4–thioglycollate method (Dische, 1947*b*), and galacturonic acid was sought by the cysteine–H_2SO_4 method (Dische, 1948).

Quantitative estimations of the glucuronic acid content of bile and of pigment B concentrates were made by the naphtharesorcinol method of Fishman & Green (1955). The amount of glucuronic acid in the purified preparation of pigment B was also determined by reduction of the alkaline-copper reagent of Schaffer & Hartmann (Peters & Van Slyke, 1932). The reagent (0·5 ml.), pigment B solution (0·5 ml., containing 800 μg. of pigment A equivalent) and 5 N-H_2SO_4 (0·2 ml.) were used, according to the directions of Hawk, Oser & Summerson (1947).

Glucuronic acid content of sera before and after preliminary alkaline hydrolysis. A volume of 0·1 N-NaOH (3 ml.) was added to serum (2 ml.) and the solution was left for 30 min. at room temperature; 0·1 N-HCl (3 ml.) and water (10 ml.) were added and mixed, followed by 10% (w/v) sodium tungstate (1 ml.) and 0·66 N-H_2SO_4 (1 ml.). After 10 min. the solution was centrifuged and 4 ml. of protein-free supernatant was used for the determination of total glucuronic acid, according to Fishman & Green (1955). A control tube of 2 ml. of serum and 16 ml. of water was similarly treated, except for alkaline hydrolysis. The bile pigments precipitated with the protein in both tubes.

Spectrophotometry. A Unicam quartz spectrophotometer (SP. 500) was used with 10 mm. quartz cells.

Paper chromatography

Conjugated bile acids. These were identified by the technique of Haslewood & Sjövall (1954). Since the main contaminants of pigment B were glycocholic and glycochenodeoxycholic acids, system G_3, consisting of a mobile phase of *iso*propyl ether–*n*-heptane (3:2, v/v) and a stationary phase of 70% (v/v) aqueous acetic acid, was most useful. Pigment A had an R_F of approx. 0·8, whereas pigment B moved with glycocholic acid (R_F less than 0·1). Pigments A and B stained blue with phosphomolybdic acid but could be identified by visual examination of the papers before spraying.

Amino acids. Pigment B was hydrolysed at 100° in 6 N-HCl for 16 hr. in a sealed tube. After electrolytic desalting and reduction of volume, the hydrolysate was examined by paper chromatography in *sec.*-butanol–formic acid–water (15:3:2, by vol.) as devised by Ahrens & Craig (1952). The pigments moved just behind the solvent front. The amino acids were demonstrated by staining with ninhydrin.

Sugars and uronic acids. Paper chromatography was carried out in *n*-butanol–acetic acid–water (4:1:5, by vol.) and freshly prepared pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.) (Fischer & Dörfel, 1955), respectively. To prepare a glucuronic acid standard, glucuronolactone was dissolved in 0·1 N-NaOH and neutralized. Aniline phthalate (Partridge, 1949) was used to stain reducing substances and a naphtharesorcinol reagent [prepared by mixing equal volumes of 0·2% ethanolic naphtharesorcinol and 2% (w/v) aqueous trichloroacetic acid immediately before use] was employed to show uronic acids and pentoses, which stained blue in the presence of moisture, while ketoses gave a red spot (Partridge, 1948).

Partition chromatography of pigments

Reverse-phase partition columns of 3 g. of silicone-treated kieselguhr in glass tubes of 15 mm. diameter were used. Bilirubin was identified by the $CHCl_3$–CCl_4–methanol (pH 6) solvent system of Cole *et al.* (1954). For the identification and separation of pigments I and II a butanol system at pH 6 was employed (Cole *et al.* 1954).

To separate the azo pigments A and B (prepared from aniline) a system was prepared from *iso*-octanol–methanol–$CHCl_3$–0·1 M acetate buffer, pH 4 (1:10:1:10, by vol.) (Norman, 1954), 2·7 ml. of the lower layer being used as stationary phase; 100–200 μg. of pigment was used. When eluted from this column pigment B was free from conjugated bile acids. When it was desired to elute pigment A as well as B, a system of $CHCl_3$–CCl_4–methanol–0·2 M acetate buffer, pH 4 (25:25:38:12, by vol.) was used; 1·5 ml. of lower layer served as stationary phase.

Countercurrent distribution

Bottles were used for small distributions through 10–15 tubes, the lower layers being moved. The transfers were made with an all-glass syringe and polythene tubing, since exposure to certain metals alters the azo pigments and turns them blue. Distributions of 40–150 tubes were made in the apparatus of Lathe & Ruthven (1951) in which the lower phases are transferred. In order to make 150 transfers in an apparatus of 50 tubes, 63 transfers were first made, after which tubes 11–32, containing most of the pigments, were

placed in tubes 0–21 of the apparatus, respectively, and the distribution was expanded to give a total of 150 transfers. Accordingly, a number of lower phases (which did not contain pigment) were removed from the apparatus without having undergone the full 150 transfers.

Solvent system A was prepared from $CHCl_3$–methanol–0·2M acetate buffer, pH 4 (32:43:25, by vol.). Solvent system B was *n*-butanol–heptane–acetic acid–water (3:7:2:8, by vol.) (Ahrens & Craig, 1952). Solvent system C consisted of *n*-butanol–heptane–acetic acid–water (3:7:1:9, by vol.) (Ahrens & Craig, 1952).

Materials and reagents

Bilirubin and alumina for chromatography were purchased from British Drug Houses Ltd. Hydroxypyrromethenecarbinol was prepared according to the method of Overbeek, Vink & Deenstra (1955*b*). Buffers were prepared according to Cole (1933). Benzenediazonium chloride was prepared according to Cumming, Hopper & Wheeler (1950); the final solution was equivalent to 92 mg. of bilirubin/ml. *p*-Aminobenzoic acid and sulphanilic acid were similarly diazotized.

Mouse-liver β-glucuronidase was prepared according to the directions of Levvy & Marsh (1952). The deposit which was soluble at 31% (w/v) and insoluble at 50% (w/v) $(NH_4)_2SO_4$ was dialysed and kept at 4°. Limpet glucuronidase was supplied by Dr R. D. Bulbrook of the Imperial Cancer Research Fund Laboratory, Royal College of Surgeons, London. It had been prepared by the method of Dodgson & Spencer (1953). Locust-crop juice, which is a rich source of enzyme (Robinson, Smith & Williams, 1953), was obtained at the Anti-Locust Research Centre, Natural History Museum, London. The activity of each preparation was determined at pH 5·2 with 0·01M phenolphthalein β-glucuronide, kindly supplied by Dr C. A. Marsh. The inhibitory effect of boiled 5mM potassium hydrogen saccharate and phenolphthalein β-glucuronide (Levvy, 1952) was demonstrated in these systems.

Preparation of pigment A

Bilirubin (250 mg.) was refluxed in 275 ml. of $CHCl_3$ for 2 hr., filtered and cooled to 5°. Ethanol (80 ml.) was added and then 3·5 ml. of benzenediazonium chloride reagent. After 2 hr., 2 ml. of 2·5N-NaOH was added, with stirring, in order to change the colour from blue to red-blue. After 16 hr. at 5° the solution was washed three times with 100 ml. of water, to remove excess of diazo reagent and acid. The $CHCl_3$ layer was divided into three portions and a brown-red pigment removed by extraction three times with an equal volume of freshly prepared saturated $NaHCO_3$. The $CHCl_3$ layers, which retained most of the red pigment, were combined and extracted with a 50 ml. and a 30 ml. portion of 0·05N-NaOH. The alkaline extracts were combined and brought to pH 3 with 5N-HCl, after which the flocculent pigmented precipitate was separated by centrifuging.

A column for absorption chromatography was prepared by mixing 20 g. of alumina with 20 ml. of 0·01N-NaOH in 85% aqueous methanol. The crude pigment A precipitate (40 mg.), dissolved in 1 ml. of the same solvent and 0·1 ml. of 0·1N-NaOH, was transferred to the top of the column. The column was eluted with 0·01N-NaOH in 85% aqueous methanol and a red band of pigment, poorly defined in front but with a sharp rear edge, was collected in two portions. The faster was shown by partition chromatography to consist almost entirely of pigment A, and the slower also contained a less polar pigment, which was probably a mixture of the methyl esters of pigment A. The first portion of pigment was made acid (approx. pH 5) with dilute acetic acid, extracted into $CHCl_3$ and taken to dryness.

Preparation of pigment B

Human hepatic bile was used as a source of pigment II since gall-bladder bile from human autopsies is usually grossly infected. It was obtained from patients in whom a T-tube had been placed in the common bile duct at operation and was collected daily for a period of 1–20 days after operation. The bile-pigment content varied from 10 to 170 mg./100 ml. of bile. The bile was adjusted to pH 6 with 10N-HCl (approx. 2 ml./l.) with vigorous mechanical stirring.

Twice the theoretical requirement of diazonium reagent was added to the bile. Further addition did not increase the yield of azo pigment, which was about 75% of the estimated amount. The pH was readjusted to 5–6 if necessary, and the bile kept at 4° until processed further.

Concentration of pigment B. Initially 10·6 l. of bile contained diazotizable pigment equivalent to 6·7 g. of pigment A. Coupling yielded 5 g. of pigment B, expressed as pigment A. Ammonium sulphate (500 g./l.) and 20 ml. of *n*-butanol/l. were added, with stirring. After standing at 4° for several hours the lower, pigment-free, aqueous layer was drawn off and discarded. The pigment was concentrated by centrifuging the remainder (1500 *g* for 15 min.), removing the aqueous part, and drying the pad of pigment over $CaCl_2$ for several days, the lumps being broken up periodically. The concentrate (186 g.) was finely ground in a mortar and extracted with 1·3 l. of methanol at 50–60° for 15 min. The mixture was allowed to settle, the supernatant decanted, and the sludge was re-extracted five times with 370 ml. portions of methanol. The residue was discarded. The supernatant fluids were combined, the amount of pigment was estimated, and a 1 ml. portion dried *in vacuo* over $CaCl_2$, and weighed. The remaining solution was reduced *in vacuo* below 40° to a thick syrup; 3·5 g. of crude pigment B (expressed as pigment A) was obtained.

Removal of lipids, pigment A, taurine-conjugated bile acids and brown pigments. This was carried out by a number of

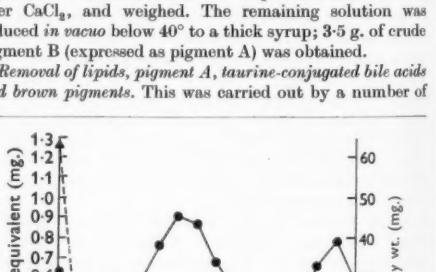

Fig. 2. Countercurrent distribution of 0·3 g. of crude pigment B through 15 tubes in solvent system A, with 225 ml. of upper phase and 15 ml. of lower phase. ●, Pigment (as pigment A); ▲, dry wt.

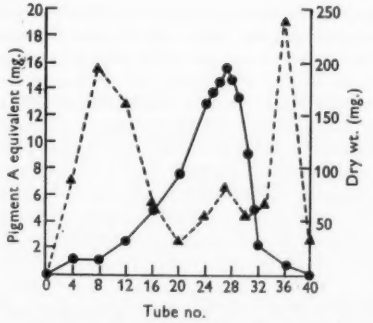

Fig. 3. Countercurrent distribution of 4 g. of pigment B concentrate through 40 tubes in solvent system B, with 24 ml. of upper phase and 36 ml. of lower phase. The distribution was begun with the solute in tubes 0–3. ●, Pigment (as pigment A); ▲, dry wt.

parallel 12–15 tube distributions in solvent system A. After a 15-tube distribution (Fig. 2), and estimation of pigment, tubes 3–10 containing pigment B were combined. Pigment B was moved into the lower phase by adding 40 ml. of aqueous saturated $(NH_4)_2SO_4$/l. of solvent. The separated $CHCl_3$, which contained almost all the pigment, was concentrated to a thick paste under reduced pressure at 40°. At this stage, the yield of pigment B was 1·7 g. (as pigment A).

Separation from glycochenodeoxycholic acid. This was obtained by several distributions in solvent system B (Fig. 3). Analysis showed that glycochenodeoxycholic acid was present in the first 20 tubes, and pigment B, together with glycocholic acid, was distributed in tubes 22–32. Tubes 22–32 were combined, and the pigment was moved into the *n*-butanol–heptane layer by adding 200 g. of $(NH_4)_2SO_4$/l. of solvent mixture. The almost colourless lower layer was discarded and most of the acetic acid was removed from the upper layer by washing four times with 0·25 vol. of half-saturated $(NH_4)_2SO_4$ and finally with 0·25 vol. of water. An equal volume of light petroleum (b.p. 40–60°) and 0·25 vol. of freshly saturated aqueous $NaHCO_3$ solution were added. The mixture was shaken and more powdered $NaHCO_3$ was added until most of the pigment had moved to the lower aqueous phase, and the pH was about 5. The aqueous phase was removed, and the upper layer again shaken with saturated aqueous $NaHCO_3$. If this took up pigment the procedure was repeated. To the combined aqueous layers one-third of the weight of $(NH_4)_2SO_4$ was added. A pigmented butanol layer separated out. This was used to form part of the solvent system for the next distribution. At this stage the yield of pigment B was 1·1 g. (as pigment A).

Separation from glycocholic acid. This was done by two parallel 150-tube distributions in solvent system C (Fig. 4). Most of the glycocholic acid was found in tubes 60–110. The tubes containing pigment B (30–59) were combined and the pigment was concentrated by the technique used after distribution in solvent B. The *n*-butanol residue was dried *in vacuo*. The final yield of pigment B was 0·65 g. (expressed as pigment A).

In the preparation from one batch of bile, a second peak of less-polar pigment was observed in a 150-tube distribution. Chromatographic examination of this material showed that it was probably a complex of pigment A and pigment B. It contained 1 molecule of glucuronic acid to 2 molecules of pigment A. Since it did not appear in subsequent distributions it was assumed to be an artifact and no further examination was made.

Precipitation of pigment B. A portion of the butanol concentrate of purified pigment B, equivalent to 56 mg. of pigment A, was added to 3 ml. of methanol, which dissolved almost all the pigment. A small amount of black deposit was washed with 1 ml. of methanol and then discarded. The methanol extracts were combined and 20 ml. of ethanol was added. A light turbidity was removed by spinning at 1500 *g* for 15 min. The supernatant was then placed in a tube at −12° for 24 hr., when a precipitate was observed. The tube was spun at 1500 *g* for 10 min. and decanted. The precipitate was dried *in vacuo* over $CaCl_2$ at room temperature to a constant weight of 18·3 mg.

Preparation of pigment I from serum

Specimens of serum from cases of obstructive jaundice were extracted with ethanol (Cole *et al.* 1954) and the resulting pigments run on partition columns. When pigment II had been eluted, the portion of the column containing the pigment I band was extracted with ethanol and the combined extracts, from several columns, were analysed for pigment and glucuronic acid.

An aqueous solution of pigment I was prepared by reducing the ethanolic solution to dryness *in vacuo*, and dissolving it in water, or by eluting the pigment I from the column and then extracting the *n*-butanol with light petroleum (b.p. 40–60°). The amounts of direct- and indirect-reacting pigment were then determined.

Effect of β-glucuronidase on pigment B

Pigment B (3 ml., equivalent to 200 μg. of pigment A) and enzyme in buffer, pH 5·2, were incubated for 1 hr. at 37° and then 7 ml. of ethanol was added. The supernatant fluid was taken to dryness *in vacuo*, and the pigmented residue was dissolved in 0·5–1 ml. of the mobile phase for partition chromatography. The bands of pigments B and A were eluted separately and estimated colorimetrically.

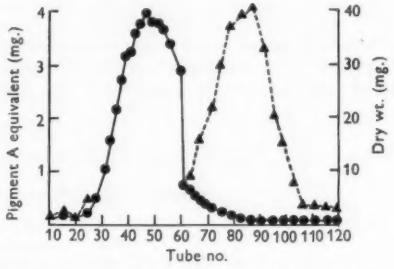

Fig. 4. Countercurrent distribution of 3·3 g. of pigment B concentrate through 150 tubes in solvent system C, with 15 ml. of upper phase and 40 ml. of lower phase. The distribution was begun with the solute in tubes 0–3. ●, Pigment (as pigment A); ▲, dry wt. The dry wt. of the contents of tubes 26–63 was not determined.

Effect of alkali on pigment B

Pigment B (equivalent to 100 μg. of pigment A) was dissolved in 3 ml. of 0·1 N-NH_3 soln. The same amount of pigment B was dissolved in 3 ml. of M phosphate buffer, pH 5, and both solutions were incubated at 37° for 16 hr. The pH was then adjusted to 4 and the solutions were taken to dryness and examined for pigments A and B by partition chromatography. Other samples were similarly treated and, after incubation and electrolytic desalting, the hydrolytic products were examined by paper chromatography for sugars and amino acids.

Effect of alkali on pigment II

Bile (1 vol.) was added to 1·5 vol. of 0·1 N-NaOH at room temperature. At varying intervals from 1 to 120 min., 0·2 ml. portions were removed and added to 4 ml. of 0·2 M acetate buffer, pH 4, containing 0·12 ml. of 0·1 N-HCl. A control was similarly treated but without the addition of alkali. Freshly diazotized 0·4% (w/v) sulphanilic acid in 0·25 N-HCl (1·4 ml.) was then added immediately to tests and control, to determine the amount of direct- and indirect-reacting pigment. The nature of the indirect-reacting pigment produced from pigment II was examined by neutralizing the mixture after 7 min., extracting the pigment with $CHCl_3$, and running it on a reverse-phase partition column, alone and with added bilirubin.

RESULTS

In preliminary experiments for determining the most satisfactory type of pigment B, the products formed by coupling pigment II with diazotized sulphanilic acid, *p*-aminobenzoic acid and aniline were examined on partition columns. All were stable and were more polar than the corresponding azo pigments formed from bilirubin. R_F values are given in Table 1. For large-scale isolation of pigment B, aniline was chosen to prepare the diazonium reagent, since the azo pigment produced was the least polar, and thus could be most easily separated from taurine-conjugated bile salts, and other polar interfering substances present in bile.

Properties of pigment B

When examined microscopically pigment B consisted of deeply pigmented spheres of remarkably uniform size, about 10 μ. diameter. In this relatively pure form pigment B was soluble in water at a reaction greater than about pH 4, soluble in methanol, but much less so in ethanol, and almost insoluble in dry *n*-butanol, chloroform, ether and light petroleum. It was stable for several weeks when dried, or at −12° in methanol, although the gradual appearance of a less polar band in partition chromatograms suggested that methylation was taking place. It was stable at pH 2 and at neutrality.

The shape of the absorption curve of pigment B in methanol did not differ appreciably from that of pigment A in the range 220–620 mμ. There were peaks of absorption at 270 and 328 mμ. as well as the main band of absorption at 517 mμ. $E_{525\,m\mu}$. of the purest preparation of pigment B was 0·57 of that calculated for pigment A (methyl red standard).

The addition of 0·1 N-NaOH (approx. pH 12) for a few minutes, or 0·1 N-NH_3 soln. (approx. pH 11) at 37° for 16 hr., changed the solubility of the pigment so that it now precipitated when the pH was adjusted to 5–6. Partition chromatography of the product showed that it moved with an R_F value identical with that of pigment A.

The aqueous products resulting from alkaline treatment of pigment B were examined for amino acids by paper chromatography. The only spot which stained with ninhydrin was faint and moved with an R_F of 0·3, like glycine. Aniline phthalate staining of paper chromatograms showed one brown spot, which moved with an R_F of 0·21, as does glucuronic acid. It stained blue with naphtharesorcinol (as do uronic acids and pentoses). Unhydrolysed pigment B moved with an R_F of about 0·9 in the system used for sugars and there was no other reducing spot.

The uronic acid which was released by alkaline hydrolysis of pigment B had an $R_{glucose}$ value of 0·36, compared with 0·34 for glucuronic acid and 0·29 for galacturonic acid, in pyridine–ethyl acetate–acetic acid. Glucuronic acid and the unknown substance stained brown with aniline phthalate, and galacturonic acid gave a pink spot. The water-soluble product of alkaline hydrolysis gave the specific carbazole–H_2SO_4 test for uronic acids. Galacturonic acid and mannuronic acid were excluded by the

Table 1. *R_F values on silicone-treated kieselguhr columns of azo pigments from bilirubin and pigment II*

Details of the partition systems are in the Experimental section.

Diazonium reagent prepared from	Partition system of *iso*-octanol–methanol–$CHCl_3$–buffer (pH 4)		Partition system of $CHCl_3$–CCl_4–methanol–buffer (pH 4)	
	R_F of pigment A	R_F of pigment B	R_F of pigment A	R_F of pigment B
Sulphanilic acid	1·0	1·0	1·0	1·0
Benzoic acid	0·3	0·8	0·7	1·0
Aniline	<0·1	0·5	0·5	1·0

mannose–thioglycollate–H_2SO_4 test, and galacturonic acid was excluded by the cysteine–H_2SO_4 test, which was negative.

Glucuronic acid content of pigment B

Pigment A gave a negative result in the naphtharesorcinol test.

The molar ratio of pigment A to glucuronic acid, in a sample of precipitated pigment B (which contained 57 % of pigment A by colorimetry), was 1·02 when the naphtharesorcinol method was used, and 0·99 with the reduction method. Calculations from their molecular weights (pigment A, 367; glucuronic acid, 196) showed that 87 % of the precipitated pigment B consisted of pigment A and glucuronic acid.

Effect of β-glucuronidase on pigment B

The β-glucuronidase preparations (from limpet, mouse liver and locust-crop juice) brought about some conversion of pigment B into pigment A, whereas the pigment in the control tube remained unaltered (Table 2).

The specific inhibitors of β-glucuronidase, boiled potassium hydrogen saccharate solution and phenolphthalein β-glucuronide, caused inhibition of the conversion of pigment B into A by limpet and mouse-liver preparations. Previous adjustment of the limpet-enzyme preparation to pH 2·25, which destroys the β-glucuronidase activity of this preparation, also destroyed its ability to change pigment B into pigment A.

The conversion of pigment B into A by locust-crop juice was not, however, inhibited by 5 mM saccharate solution, although this inhibitor was found to be effective in reducing the hydrolysis of phenolphthalein β-glucuronide. The effect of 3 mM phenolphthalein β-glucuronide as a competitive inhibitor was variable; in one experiment 34 % inhibition was observed, but in two other experiments there was no inhibition. Boiled locust-crop juice had no effect on pigment B.

Glucuronic acid content of bile

Eighty-six specimens of fistula bile (none of which was used for the above preparation) were collected from fifteen patients and analysed for direct and indirect pigments and for glucuronic acid. The relation between the content of direct-reacting pigments and of glucuronic acid is shown in Fig. 5. In twenty-six of these specimens (seven from one patient) the molar ratio of glucuronic acid to direct pigment was 2 or more. Forty-three specimens had molar ratios between 1·5 and 2.

Effect of alkali and β-glucuronidase on pigment II

Since pigment B was alkali-labile, the effect of alkali on pigment II was studied by following the change in the amount of direct and indirect pigments after 1 vol. of bile had been added to 1·5 vol. of

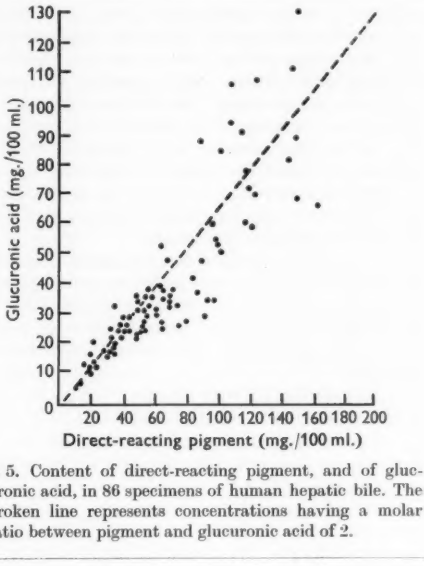

Fig. 5. Content of direct-reacting pigment, and of glucuronic acid, in 86 specimens of human hepatic bile. The broken line represents concentrations having a molar ratio between pigment and glucuronic acid of 2.

Table 2. *Effect of β-glucuronidase preparations on pigment B*

Enzyme preparation	Conversion into pigment A (%)	Inhibition (%)
Limpet	54	—
After acid treatment (pH 2·25)	Nil	100
+5 mM saccharate	6	89
+3 mM phenolphthalein β-glucuronide	23	57
Mouse liver	17	—
+5 mM saccharate	Nil	100
+3 mM phenolphthalein β-glucuronide	6	65
Locust-crop juice	86	—
After heat treatment	Nil	100
+5 mM saccharate	84	2
+3 mM phenolphthalein β-glucuronide	57–86	0–34

0·1 N-NaOH at room temperature (Fig. 6). The direct-reacting pigment was rapidly transformed into approximately an equivalent amount of indirect-reacting pigment. This was identified on a partition column as bilirubin. The breakdown of bilirubin, which is formed in this way, could be explained by the presence of the bile, which had a similar effect on the pure compound under these conditions.

Fig. 6 also shows that a small part of the direct-reacting pigment was alkali-stable, as indicated by the relatively constant amount of direct-reacting pigment present after 30 min. of alkali treatment. The amount of this component varied from 5 to 40 % of the whole in different bile specimens, and was usually 10–15 %. It was not proportionately elevated in those specimens which contained high concentrations of total pigment.

It is possible that another pigment may be present in some specimens of bile, for alkaline treatment of the hepatic bile of six patients during the immediate period after operation revealed a marked drop in the direct-reacting component without a commensurate rise in the indirect one. In three cases the more usual behaviour, represented in Fig. 6, had returned by post-operative days 5–7, but in two cases the unusual behaviour was still present, though reduced in amount, at days 9 and 11. This effect may have been due to a greatly enhanced breakdown of bilirubin.

It was not possible to determine the effect of mouse-liver β-glucuronidase on the pigment of fistula bile, since it was observed that bile markedly inhibited the activity of the enzyme when phenolphthalein β-glucuronide was used as substrate.

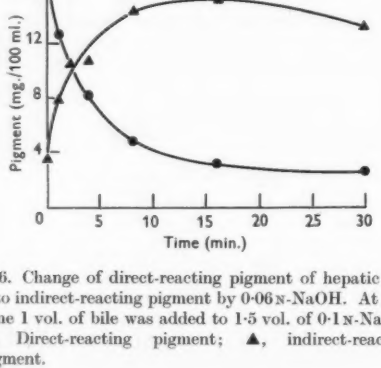

Fig. 6. Change of direct-reacting pigment of hepatic bile into indirect-reacting pigment by 0·06 N-NaOH. At zero time 1 vol. of bile was added to 1·5 vol. of 0·1 N-NaOH. ●, Direct-reacting pigment; ▲, indirect-reacting pigment.

Pigment I

In pigment I which had been separated chromatographically the molar ratio of pigment (expressed as bilirubin) to glucuronic acid was found to vary from 0·8 to 1·2. This variability is probably due to the low concentrations of pigment available for the determinations. Aqueous solutions of pigment I gave a direct reaction in the van den Bergh test. The addition of ethanol to the reaction mixture after 30 min. increased the yield of azo pigment by approx. 40 %.

Alkali-labile glucuronides of serum

The glucuronic acid content of sera from six patients with obstructive jaundice (containing pigments I and II) and three babies with haemolytic disease (whose predominant serum bile pigment was bilirubin) was compared with that of sera from five normal persons. This was done with and without preliminary alkaline hydrolysis of the serum, since without hydrolysis the glucuronic acid component of pigments I and II would be removed because of the affinity of direct-reacting pigments for precipitated plasma proteins. Treatment with alkali increased the apparent glucuronide content of sera from haemolytic patients, and from normal persons, by less than 0·5 mg./100 ml. With sera from patients with obstructive jaundice (containing varying amounts of pigments I and II) the increase ranged from 1·7 to 3·6 mg./100 ml.

DISCUSSION

Coupling of bilirubin and pigment II with diazonium compounds

Fischer & Haberland (1935), from their observations on the dimethyl esters of mesobilirubin IIIα and XIIIα, concluded that the coupling of bilirubin with diazonium compounds involved a preliminary rupture of the methene bridge with the resulting production of pigment A and hydroxypyrromethenecarbinol. Overbeek *et al.* (1955*b*) showed that, in the presence of sufficient coupling agent, the hydroxypyrromethenecarbinol was converted into pigment A and thus that 2 molecules of pigment A are formed from 1 molecule of bilirubin. Since pigment II forms only one pigment in this coupling reaction it can be concluded, by analogy, that 1 molecule of pigment II will form 2 molecules of pigment B. Pigment B has been shown to be a derivative of pigment A and it is, therefore, proposed that the formation of pigment II involves a similar substitution in each half of the bilirubin molecule.

Structure of pigment B

Proof that pigment B was the ester glucuronide of pigment A depended on the observation that under mild alkaline conditions, in which ester glucuronides are labile (Williams, 1947), pigment B yielded pigment A and glucuronic acid. The latter was identified by chromatography and by specific tests. The molar ratio of pigment A to glucuronic acid was approximately 1. β-Glucuronidase preparations from limpet, locust juice and mouse liver also hydrolysed pigment B to pigment A (Table 2). The specificity of this enzyme activity was shown, in the mouse and limpet preparations, by inhibition of the enzymic hydrolysis with boiled saccharate and phenolphthalein β-glucuronide. Conversion of pigment B into pigment A by the limpet preparation was also stopped by prior exposure to pH 2·25; this treatment inhibits β-glucuronidase but not aryl sulphatase activity (Dodgson & Spencer, 1953). With the locust enzyme the lack of inhibition by saccharate was unexplained, as the activity of this enzyme preparation against phenolphthalein β-glucuronide was inhibited by saccharate. With this exception all the evidence suggested that pigment B was an ester glucuronide of pigment A.

The quantitative analysis of the purest pigment B preparation indicated that it was 87 % pigment A glucuronide. The remaining impurity was probably glycocholic acid, since the amount of glycine observed on paper chromatography was consistent with about 15 % contamination with this substance. This was not surprising in view of their similar behaviour in countercurrent distributions (Figs. 3, 4) and in paper chromatography (system G_3 of Haslewood & Sjövall, 1954).

Structure of pigment II

The finding that pigment B was the glucuronide of A suggested that pigment II was bilirubin diglucuronide. A monoglucuronide structure was excluded since coupling of pigment II in small-scale trials yielded only pigment B, and not equal amounts of A and B as a monoglucuronide structure would have required. Under mild alkaline conditions pigment II produced bilirubin, as shown by its chromatographic behaviour and indirect van den Bergh reaction (Fig. 6). The glucuronic acid content of hepatic-bile specimens was usually a little less than that necessary for conjugation with both carboxyl groups of all the direct-reacting pigment (Fig. 5). However, a variable amount of alkali-stable direct-reacting pigment was observed in all specimens (Fig. 6). If this was not a glucuronide, as appears probable, it may account for the frequent occurrence of a minor deficit in the glucuronic acid content of bile.

Theoretically it was conceivable that pigment II was a dipyrrole pigment which combined to form bilirubin. Fischer & Haberland (1935) considered that some dipyrroles could yield bilirubinoid pigments. However, there is no direct evidence that bilirubin is excreted as a dipyrrole compound. Moreover, the production of bilirubin from pigment II by alkaline hydrolysis was strong evidence that the latter was also a tetrapyrrole pigment, and that at the central methene group it had the same structure as bilirubin.

The yield of pigment B in the large-scale preparation was less than 5 % of that contained in the original bile. This is due mainly to such factors as incomplete conjugation, relatively poor extraction with methanol from the dry extract, the degradation of pigment B to A during the various procedures (most noticeable in Fig. 2), and the discarding of countercurrent tubes containing some pigment B, together with large amounts of impurities. The possibility that larger amounts of some other azo pigment similar to B was removed during the preparation has had to be considered. However, partition chromatograms of the original diazotized biles showed only one type of pigment B and, at all stages in the preparation, analyses of specimens of pigment B obtained from peak tubes of countercurrent distributions indicated a molar ratio of approximately 1 for pigment to glucuronic acid. There was no evidence that the original pigment B contained an appreciable amount of non-ester glucuronide component, as might have arisen from the alkali-stable direct pigment shown in Fig. 6. Thus the low yield of pigment B is not inconsistent with bilirubin diglucuronide being the main pigment of human bile.

It would have been more satisfactory if the postulated structure for pigment II could have been supported by the results of direct glucuronic acid analysis of a band of pigment II from a partition column, or in a countercurrent distribution. However, it has not yet been possible to develop a satisfactory partition column for this purpose, and the analysis of small countercurrent distributions of bile gave extremely variable results. It was uncertain how much of this was due to the breakdown of pigment II during the distribution, or to irregular behaviour as a result of complex formation with bile salts. Attempts to show that liver β-glucuronidase changed the direct-reacting pigment of bile to an indirect one were also unsuccessful. This was attributed to the presence of a β-glucuronidase inhibitor in bile, as it was found that the addition of bile inhibited the hydrolysis of phenolphthalein β-glucuronide by mouse-liver enzyme. The occurrence of natural inhibitors of β-glucuronidase in tissue extracts has previously been noted (Levvy, 1953).

Although further work is necessary to clarify some of these aspects, the evidence indicates that bilirubin is excreted by the liver as a diglucuronide. The alkali lability suggests that the linkage is of an ester type, the propionic acid side chains of bilirubin linking with the $C_{(1)}$ hydroxyl of glucuronic acid. A number of aromatic acids and aliphatic branched acids are excreted in the urine as glucuronides of this type (Kamil, Smith & Williams, 1953), nicotinic acid (van Eys, Touster & Darby, 1955), aspartic and glutamic acids (Pollack & Eades, 1954). Glucuronides of thyroxine (Taurog, Briggs & Chaikoff, 1952) and of oestriol (Kinsella, Francis, Thayer & Doisy, 1956) probably occur in bile.

The same conclusion has been reached independently by Talafant (1956*a*), who showed that, in paper electrophoresis of bile, pigment II was constantly associated with glucuronic acid in a molar ratio 1:2. β-Glucuronidase preparations yielded bilirubin. The lead salt of bilirubin diglucuronide has also been prepared (Talafant, 1956*b*). Schmid (1956) has also presented paper-chromatographic and enzymic evidence that pigment B is the glucuronide of pigment A, but has suggested that the linkage is of the glycosidic type.

Pigment I

Pigment I yields both pigment A and B in the diazo reaction (Billing, 1954). This is consistent with a monoglucuronide structure. In determinations of the molar ratio of glucuronic acid to pigment I a value of about 1 was found. The possibility that pigment I is a complex of bilirubin and pigment II has had to be considered in view of our previous report that pigment I could be converted into bilirubin and pigment II by warming and evaporating its solutions and rechromatographing them. The material available for study has been limited, and some but not all of the specimens have shown this transformation. The present evidence therefore favours a monoglucuronide structure.

Although pigment I couples directly in the van den Bergh test, it should yield some hydroxypyrromethenecarbinol. We have found that the coupling of hydroxypyrromethenecarbinol with diazonium compounds requires the presence of ethanol for its conversion into pigment A. Accordingly, it was to be expected that the yield of azo pigments formed would be increased by the addition of ethanol. In a few trials the increase amounted to about 40%. This would also result from mixtures of bilirubin and pigment II.

Differences between bilirubin and pigment II

A number of differences between direct- and indirect-reacting pigments have been noted in the past, especially with regard to solubility, occurrence in urine, dialysability, affinity for serum protein and protein precipitates, ease of oxidation, affinity for brain tissue and association with different types of jaundice. Some of these differences can be explained on the basis of conjugation with glucuronic acid.

A difference in solubility of direct- and indirect-reacting pigments has been known for some time. Bilirubin is lipid-soluble (approx. 0·1% in chloroform), and its solubility in water at neutrality is negligible (0·0001%) according to Overbeek, Vink & Deenstra (1955*a*). In contrast, pigment II is insoluble in chloroform and soluble in water. It occurs in bile in concentrations up to 0·4%. It is well known that conjugation with glucuronic acid greatly increases the water solubility of many substances.

The insolubility of bilirubin in water at neutrality probably explains why bile pigments do not appear in the urine in those conditions in which bilirubin is the chief serum pigment (acholuric jaundice, haemolytic jaundice). In obstructive jaundice and hepatitis, however, pigments I and II occur in the serum. These pigments are water soluble and can therefore be excreted in the urine. Hitherto the occurrence of direct-reacting pigments in the urine has been attributed to their greater dialysability. In our experience both types will dialyse slowly if the reaction is such that they are soluble. Under physiological conditions the decisive factor determining the dialysability of direct pigments and the non-dialysability of bilirubin is the solubility of the former and the insolubility of the latter in aqueous solution.

It has been suggested that 'indirect' and 'direct' bilirubin are attached to different plasma proteins, and that this explains their difference in behaviour. Gray & Kekwick (1948) found that both were attached to albumin. A report by Najjar (1952) based on salting-out techniques, and indicating that direct-reacting pigment has an affinity for albumin, and indirect-reacting pigment for globulin, was not confirmed by Klatskin & Bungards (1956). Nevertheless, denatured protein, as obtained by ethanol precipitation, has a greater affinity for direct-reacting pigments than for bilirubin. This may also be partly a question of solubility, but some other unknown factor is probably involved.

A striking difference between the two types of pigment is shown by a greater ease of oxidation of direct-reacting pigments in body fluids. The greater stability of bilirubin may be partly due to its association with lipids which exert an antioxidant action. The lipids in bile seem to protect pigment II in the same way, since removal of lipid with chloroform is followed by rapid oxidation. The affinity of bilirubin for brain tissue (Claireaux, Cole & Lathe, 1953), and its toxic effect, probably also depend on its lipophilic characteristic, as the water-soluble direct-reacting pigments do not stain brain tissue,

and are non-toxic. In this respect the behaviour of bilirubin is similar to that of morphine, which is also lipid-soluble (and water-insoluble) and is excreted as a water-soluble glucuronide, which has no effect on the brain (Woods, 1954).

The distinction between direct and indirect reactions in the van den Bergh test is probably also determined by the different solubility of the pigments, as ethanol has to be added only to the pigment which is insoluble in water (at the acid reaction of the test). However, bilirubin will couple directly in the absence of ethanol if the reaction is alkaline (Gedigk & Gries, 1952), since bilirubin then exists as a soluble salt. The importance of solubility is also born out by the recent work of Jirsa, Večerek & Ledvina (1956), who prepared the mono- and ditaurine conjugates of bilirubin. These synthetic pigments, which are also soluble at an acid reaction, behave like the natural bilirubin conjugates (pigments I and II) in partition chromatography and electrophoresis, in regard to ease of oxidation, and in the van den Bergh reaction.

The association of direct-reacting serum pigments with obstructive rather than haemolytic jaundice suggests that this pigment can be formed only in the liver. *In vitro* studies indicate that only liver tissue has a high capacity to form glucuronides (Karunairatnam, Kerr & Levvy, 1949). The kidney was found to have only one-quarter of this activity. Bollman & Mendez (1955) and Bollman (1956) noted that in the hepatectomized dog there was a slow conversion of bilirubin into pigment I. As pigment II of dog bile is also bilirubin diglucuronide (our unpublished observations) this suggests that *in vivo* the capacity to conjugate with glucuronic acid is not entirely confined to the liver, and perhaps the formation of the monoglucuronide may be effected by the kidney.

SUMMARY

1. Pigment II, the direct-reacting pigment of human bile, when coupled with benzenediazonium chloride, forms azo pigment B, whereas bilirubin forms azo pigment A. A comparison of the properties of these two azo pigments indicated that pigment B is the ester glucuronide of pigment A.

2. It has been shown that bilirubin is excreted in human bile as an ester diglucuronide.

3. The other direct-reacting bile pigment, pigment I, which occurs in serum and urine together with pigment II, is probably an ester monoglucuronide of bilirubin.

4. The difference in the type of van den Bergh reaction, and in the physiological behaviour of bilirubin and pigments I and II, can be accounted for by the fact that bilirubin is lipid-soluble, whereas the bilirubin glucuronides are water-soluble.

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[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **356th Meeting** *of the Biochemical Society was held at the Institute of Psychiatry, University of London, Maudsley Hospital, on Saturday,* 17 *November* 1956, *starting at* 2 *p.m., when the following papers were read:*

COMMUNICATIONS

The Turnover of Brain Phospholipids during Anaesthesia, Chlorpromazine Sedation and Convulsions.

By G. B. Ansell and H. Dohmen. (*Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff*)

Experiments with [^{32}P]phosphate have shown that the metabolism of the brain phospholipids as a whole responds to changes in the physiological state (Dawson & Richter, 1950). In the present study the effect of thiopentone anaesthesia, the tranquillizing drug chlorpromazine and electrically induced convulsions on phospholipid metabolism in the rat brain was investigated. Specific activities of individual phospholipids were measured by the method of Dawson (1954). The ratio of the specific radioactivity of the phospholipid–phosphorus to that of the acid-soluble phosphorus 3 hr. after the injection of [^{32}P]phosphate was used as an index of phospholipid turnover.

Mean specific activity ratios ($\times$ 1000) of 558 $\pm$ S.D. 104 for diphosphoinositide, 54 $\pm$ 5 for phosphatidyl choline, 29 $\pm$ 4·9 for phosphatidyl ethanolamine and $<$ 8 for phosphatidyl serine were obtained for normal animals. A water-soluble, phosphorus-containing compound, appearing on two-dimensional chromatograms and deriving from an unidentified lipid by the method of Dawson (1954), gave a ratio of 136 $\pm$ 20, intermediate between that of the diphosphoinositide and phosphatidyl choline.

Deep thiopentone anaesthesia caused a reduction in total phospholipid turnover of 73 %, whilst phosphatidyl choline and phosphatidyl ethanolamine turnovers were reduced by 80 and 67 % respectively. No significant change was observed in the phosphatidyl serine, but its uptake of ^{32}P was low and difficult to determine accurately.

Chlorpromazine (20 mg./kg. body weight) reduced the phospholipid turnover by 56 %. The turnover values for phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine, were reduced by 63, 50 and *ca.* 63 % respectively. There was also a considerable reduction in diphosphoinositide turnover. Phospholipid turnover was uniformly reduced in the cerebral cortex, white matter, medulla, pons and cerebellum. These results may be contrasted with those of Wase, Christensen & Polley (1956). The reduced uptake of ^{32}P into the brain phospholipids in chlorpromazine-treated animals may be related to the observation that the drug uncouples oxidative phosphorylation in brain tissue *in vitro* (Abood, 1955). Electrically induced convulsions produced little or no change in the uptake of labelled phosphate into the different phospholipid fractions (cf. Dawson & Richter, 1950; Torda, 1954).

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The Effect of the Persistence of the Inhibitor on the Rate of the Return of Cholinesterase Activity *in vivo* after Injection with Different Dimethyl Phosphate Esters.

By M. Vandekar. (*Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey*)

Aldridge (1953) showed that cholinesterase inhibited by certain dimethyl phosphate esters *in vitro* recovers its activity more rapidly than when the corresponding diethyl esters are inhibitors. It was shown later that inhibition by organophosphorus compounds is a two-stage chemical process (Wilson, 1955), the initial form of the inhibited enzyme being converted at a first-order reaction rate into a second form which can no longer be reactivated (Davies & Green, 1956). This conversion occurs rapidly with dimethylphosphorylated cholinesterase and evidence is here presented that the same two-stage process occurs *in vivo*.

Marked differences in the duration of symptoms were observed when sublethal single doses of different dimethyl phosphate esters were injected

intravenously into rats. The compounds inducing prolonged symptoms (*O*, *O*-dimethyl *S*-ethylthioethylphosphorothiolate, *O*, *O*-dimethyl *S*-ethylsulphinylethylphosphorothiolate and *O*, *O*-dimethyl *S*-ethylsulphonylethylphosphorothiolate) produced an inhibitor (which may not be the parent compound) which persisted for several hours in the circulating blood. This was shown by testing the inhibitory power of serum at different intervals after injection. Small amounts of inhibitor were detectable even after 24 hr. In contrast those compounds inducing symptoms of short duration (*O*, *O*-dimethyl *S*-ethylsulphonioethylmethylphosphorothiolate and dimethyl *p*-nitrophenylphosphate) did not show any persistent inhibitor in the blood.

There was no distinction between the persistent and non-persistent inhibitors when tested *in vitro* for the return of activity by Aldridge's method—the inhibitor and enzyme are left in contact for only 15 min. The return of activity took place at the same high rate in all instances (half-life *ca.* 1·3 hr.).

In vivo the rate of return of brain and erythrocyte cholinesterase activity differed markedly between the two groups of inhibitors: the persistent inhibitors produced irreversible inhibition of practically all of the inactivated enzyme, whereas most of the enzyme inhibited by the non-persistent inhibitors recovered its activity rapidly. It has further been shown that a non-persistent inhibitor (dimethyl *p*-nitrophenylphosphate) will produce irreversible inhibition if the inhibitor is made persistent by slow infusion for several hours into the rat's jugular vein.

The return of cholinesterase activity after administration of dimethyl phosphate esters depends both on the persistence of the inhibitor in the body and on the route and/or speed by which the inhibitor is administered.

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Glutathione and Glutathione Reductase in Cerebral Tissues. By H. McIlwain, H. Martin and M. A. Tresize. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

Glutathione has been determined in the brain of animals rapidly frozen *in situ* with liquid nitrogen and also in separated cerebral tissues incubated in oxygenated glucose salines. Modification of a glyoxalase method (Patterson & Lazarow, 1955) enabled the oxidized and reduced forms to be determined, giving (μmoles/g., with S.D.) for rat brain, *in situ*: oxidized, $0{\cdot}05 \pm 0{\cdot}10$; reduced, $3{\cdot}34 \pm 0{\cdot}23$. In guinea-pig brain, *in situ*, were found oxidized, 0·05; reduced, $2{\cdot}61 \pm 0{\cdot}4$. After 60 min. incubation, tissue slices showed: oxidized $0{\cdot}42 \pm 0{\cdot}06$; and reduced, $1{\cdot}42 \pm 0{\cdot}17$.

Among factors conditioning the persistence of oxidized glutathione *in vitro*, glutathione reductase appears outstanding, for when operating under optimal conditions it is capable of reducing, by reduced 2′-phosphocozymase, over 200 μmoles of oxidized glutathione/g. tissue/hr. (McIlwain & Tresize, 1956). Study of its requirements has shown half-maximal rates to be reached under otherwise optimal conditions when reduced 2′-phosphocozymase is at about 6 μM and oxidized glutathione at about 50 μM. Reduced phosphocozymase is reported as present at 11 ± 4 μmoles/kg. in rapidly frozen rat brain (Glock & McLean, 1955) and about twice that level in the guinea pig. This is consistent with the low level of oxidized glutathione in such tissue. Whether the persistence of much more oxidized glutathione in the incubated tissue implies a different balance in oxidized and reduced forms of the nicotinamide coenzyme is not known. Until stimulated, the tissue's respiration is lower *in vitro* than *in vivo*, which would not in itself be expected to lead to change in the direction observed.

Conditions modifying the tissue's glutathione are being studied. Changes in blood level of glutathione have been associated with mental changes (see Altschule, Siegel & Henneman, 1952; Altschule, Siegel, Goncz & Murnane, 1954).

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Phosphates from Cerebral Tissue which Increase in Radioactivity in the Presence of $^{32}PO_4$ during the Passage of Electrical Pulses. By P. J. HEALD. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E. 5*)

It has been shown (Heald, 1956) that during the brief passage of electrical pulses through slices of cerebral tissues which had been metabolizing radioactive phosphate, there was a decrease in the specific activity (counts/min./μg. P) of the phosphates soluble in trichloroacetic acid and an increase in the specific activity of the extracted residue. Fractionation of the tissue residue by the method of Strickland (1952) has shown that the increase occurs largely in the phosphorus considered to be derived from 'phosphoprotein'. There is a slight increase in activity of the phospholipid fraction but this is not statistically significant.

Attempts have been made to isolate such fractions from the tissue by methods less degradative than those mentioned above. Tissues are initially fixed and extracted with chloroform–methanol (2:1, v/v) and after washing with saturated ammonium sulphate at pH 7·4, are extracted with 0·3 M ammonia. Fractionation of the ammonia extract on Dowex-1 chloride yields two fractions, A and B. Fraction A contains much contaminating inorganic phosphate. Fraction B shows an increase in specific activity when derived from tissues to which pulses have been applied. This fraction is composite and contains guanosine nucleotides.

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Porphyrin Biosynthesis in Human Red Cells. By C. RIMINGTON and H. L. BOOIJ. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1*)

Since normal human red cells are unable to synthesize haem from added glycine, it has been assumed that they lack the power to accomplish any of the stages of haem biosynthesis. Falk and Dresel (personal communication) observed the formation of porphyrins from porphobilinogen (PBG) by human haemolysates, and Fallot, Canivet, Mondet & Poidatz (1956) have recently claimed that in N_2 this system forms uroporphyrin I from δ-aminolaevulic acid (ALA).

Our studies of human red-cell haemolysates show that at non-limiting concentrations of ALA, uroporphyrin (series III + *pseudo*-uroporphyrin) is formed in large amount together with a lesser quantity of coproporphyrin III and a very small amount of protoporphyrin. The presence of a suspension of rat-liver mitochondria in the system enables biosynthesis to proceed to the protoporphyrin stage, the amount of this porphyrin formed increasing greatly and that of uroporphyrin and coproporphyrin decreasing (Rimington, 1956). ALA and PBG are qualitatively and quantitatively equivalent in the human red-cell system as they are in the chicken haemolysate. Uroporphyrinogen III can also be utilized and is transformed into coproporphyrin III.

The enzyme systems concerned with these transformations are relatively stable. Freshly prepared haemolysate may be frozen and thawed with negligible loss of activity. Haemolysates have been prepared at intervals from human blood kept at 2°; there is a progressive diminution in activity (towards ALA at 37°) which is not restored by treatment of the blood with adenosine. Even after 3–4 weeks significant quantities of porphyrins are formed and these are still series III isomers. Heating of whole blood, washed cells, haemolysate or supernatant from the latter, before addition of substrate and incubation at 37°, profoundly alters the systems, as is described in the following communication. Among other findings is the formation of series I porphyrins in place of the usual series III. In congenital porphyria, a gene-controlled metabolic disturbance of the haemopoietic system, quantities of uroporphyrin I and coproporphyrin I are excreted. We have found that haemolysate prepared from a case of congenital porphyria produces considerable quantities of uroporphyrin I and coproporphyrin I, together with the III series isomers.

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Effect of Pre-heating on Porphyrin Synthesis by Red Cells. By H. L. Booij and C. Rimington. (*Department of Chemical Pathology, University College Hospital Medical School, London, W.C.* 1)

Whole chick or human red cells, their haemolysates and supernatants derived from the latter, normally form series III porphyrins from δ-aminolaevulic acid (ALA) or porphobilinogen (PBG) at 37°. If, however, these systems are pre-heated (e.g. at 60° for 0·5 hr.) before addition of substrate, the biosynthesis is modified in two ways, (1) the yield of uroporphyrin is greatly increased, that of coproporhyrin and protoporphyrin decreased, and (2) uroporphyrin I and coproporphyrin I replace the III series isomers. The extent of these changes is dependent upon time and temperature of pre-heating. Aged blood or haemolysate responds to pre-heating as does fresh blood.

There is some evidence of partial restoration to normal by addition of unheated to the pre-heated haemolysate, in that yields of uroporphyrin are lower and of coproporphyrin higher than expected if each portion acted independently. Moreover, in a 1:1 mixture uroporphyrin III is the predominant isomer and coproporphyrin is entirely of the III series. No restorative effect of this kind is exerted by normal plasma or by ATP or an ATP-generating system. Chick blood appears to be somewhat more resistant to pre-heating than human blood but, nevertheless, the same changes have been observed to occur. Human and avian systems aged at 2° still give rise to series III porphyrins at 37° and to uroporphyrin I when pre-heated. The nature of the change caused by heating is not yet clear. *Chlorella* preparations behave somewhat similarly (Bogorad & Granick, 1953), pre-heating at 55° causing formation of I instead of III series porphyrins from PBG. This change also takes place during the course of a 24 hr. incubation at 37°. Fallot, Canivet, Mondet & Poidatz (1956) claim that human red-cell haemolysates incubated for 24 hr. with ALA in N_2 form uroporphyrin I. Preliminary observations suggest that this effect may be different from that caused by pre-heating.

These investigations are of importance toward an understanding of the metabolic defect in congenital porphyria. Haemolysate of congenital porphyria blood incubated with PBG at 37° yields a mixture of I and III series porphyrins, so the defect is present in some, at least, of the circulating cells and is not confined to processes occurring in the bone marrow during early maturation of the erythrocytes.

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Metabolism of Uniformly ^{14}C-labelled Sorbitol in Man. By L. H. Adcock and C. H. Gray. (*King's College Hospital Medical School, Denmark Hill, London, S.E.* 5)

When 35 g. sorbitol is fed to normal and mildly diabetic human subjects, negligible concentrations of sorbitol appear in the blood and urine, and even in the diabetic subject there is only a minor increase in blood sugar concentrations.

When [U-^{14}C]sorbitol was fed to normal subjects and a mild diabetic, at least 75 % of the radioactivity was recovered in the expired CO_2 within 24 hr. Little radioactivity appeared in the urine or faeces. In a more severe diabetic excreting glucose in the urine, increased radioactivity appeared in the urine and only 65 % of the radioactivity was recovered in the expired CO_2. The blood glucose was radioactive within 1 hr. of the ingestion of [U-^{14}C]sorbitol indicating a rapid conversion of sorbitol into glucose.

Comparison of the results with those obtained with a similar dose of [U-^{14}C]glucose suggested that although a substantial proportion of oral sorbitol is metabolized via glucose, a significant proportion is probably metabolized via a more direct pathway.

Separation of the Chondroitinase and Chondrosulphatase Systems of *Proteus vulgaris*. By K. S. Dodgson and A. G. Lloyd. (*Department of Biochemistry, University of Wales, Newport Road, Cardiff*)

After testing a number of bacteria for the presence of chondrosulphatase a strain of *Proteus vulgaris* was shown to be a particularly potent source of the enzyme. Chondrosulphatase has been obtained in a soluble form from the acetone-dried cell preparation and has been concentrated thirty times (Dodgson, Lloyd & Spencer, 1957). In common with the chondrosulphatase of other bacteria (Neuberg & Hofmann, 1931) and molluscs (Soda & Egami, 1938), the *Proteus* enzyme is closely associated with a mucopolysaccharase or chondroitinase which degrades the polysaccharide chain of chondroitin

sulphate with the release of reducing substance. Using an enzyme preparation containing chondroitinase it was found that chondrosulphatase did not hydrolyse the substrates of aryl-, glyco-, myro- and steroid sulphatases, nor did it attack a number of polysaccharide sulphate esters including heparin, carragheenin, fucoidin and synthetic laminarin sulphate. Uridine diphosphate acetylgalactosamine sulphate (Strominger, 1955) and the acetylgalactosamine sulphate derived from this compound were not hydrolysed (Dodgson *et al.* 1957).

The present communication describes the first successful attempt to obtain chondrosulphatase free from chondroitinase by selectively adsorbing the former on calcium phosphate gel (Keilin & Hartree, 1938) under rigorously controlled experimental conditions. The chondrosulphatase prepared in this way, in the absence of chondroitinase, shows only a slight sulphatase activity towards the polymerized form of chondroitin sulphate but exhibits considerable activity towards the sulphated products obtained following the exhaustive degradation of chondroitin sulphate by testicular hyaluronidase. At the present time it has not been possible to obtain chondroitinase completely free from chondrosulphatase. However, the latter enzyme can be completely inhibited by a brief pre-incubation with 0·01 M phosphate without affecting the ability of chondroitinase to degrade the polysaccharide chain of chondroitin sulphate.

It would appear, therefore, that hydrolysis of ester sulphate groups is not an essential pre-requisite for chondroitinase activity. On the other hand, chondrosulphatase activity is greatly enhanced by preliminary degradation of the polysaccharide chain.

One of us (A. G. L.) is grateful to the Medical Research Council for a Studentship.

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Bracken Poisoning of Cattle—Nature of the Poison. By A. J. Thomas, I. A. Evans and W. C. Evans. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Bracken poisoning in cattle is characterized by haemorrhage and pyrexia resulting from a severe leucopenia and thrombocytopenia, due to depression of bone-marrow activity (Evans, Evans & Hughes, 1954). It has been tentatively suggested that the toxic substance is a water-soluble small molecular substance (Thomas, Watkin, Evans & Evans, 1955). Steaming of bracken abolishes its toxicity, so that the substance is presumably heat-labile, or liberated by an enzyme. To investigate this further, finely milled low-temperature-dried bracken, in 100 lb. lots, was exhaustively extracted at room temperature with (*a*) acetone, (*b*) ether and (*c*) water. The solvent phases were separated from the bracken residues and concentrated under reduced pressure. The dry residues from these extractions were fed to three yearling Welsh Black cattle, at the rate of 5 lb. per day, mixed with bran; the rest of the diet was hay. The acetone, ether, and water extracts, each equivalent to 5 lb. milled bracken, were given by rumen fistula to another three animals, daily.

The cattle receiving the acetone and ether extracts of bracken showed normal values for all blood elements, and no poisoning developed. By contrast, the cattle receiving the residues developed clinical bracken poisoning within a month. Thus, the agent responsible for suppression of bone-marrow activity is not soluble in either acetone or ether. The residues still contained thiaminase, the activity being unimpaired by these solvent treatments.

The cattle receiving the water extract and residue gave equivocal results. The residue produced no significant change in the blood elements. The water extract led to a greater fluctuation in leucocytes and platelets than is usual with normal animals, but at no time did they depart from the normal range. A possible explanation would be that only partial extraction of the poison was achieved with water, resulting in a division of activity, so that the toxic dose was not reached in either animal. Further experiments are in progress to clarify this.

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Bracken Poisoning of Cattle—Therapeutic Treatment. By W. C. EVANS, I. A. EVANS, C. M. EDWARDS and A. J. THOMAS. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Marberg & Wiles (1938) reported that extracts of yellow bone-marrow were of value in the treatment of agranulocytosis in humans. The non-saponifiable fraction of the active extract was shown by Holmes, Corbet, Geiger, Kornblum & Alexander (1941) to contain D-batyl alcohol (D-α-octadecylglycerol-ether). Since then, this alkoxy-glycerol has been found in spleen, liver and aorta (Prelog, Ruzicka & Stein, 1943). In 1949, Sandler reported a protective action of bone-marrow extracts in rats poisoned with benzene, which he repeated with batyl alcohol. Edlund (1954) claimed that injection of DL-batyl alcohol increased the survival time of mice given total body X-irradiation. According to Brohult & Holmberg (1954) a striking response is obtained when this substance is administered to patients suffering from irradiation leucopenia.

Since bracken poisoning in cattle produces bone-marrow damage, similar to that produced by radiomimetics, etc., and is almost invariably fatal, it was decided to test the efficacy of DL-batyl alcohol in this condition.

Bracken poisoning was produced in two Welsh Black bullocks. When the clinical symptoms of haemorrhages and pyrexia appeared, one animal was injected subcutaneously with DL-batyl alcohol (1 g. in 10 ml. olive oil) for 5 consecutive days. The other animal received olive oil alone. Both animals were given comprehensive antibiotic therapy. After 3 days, the animal receiving no batyl alcohol died; the other animal showed a leucocyte response, the pyrexia abated, and the animal recovered. In another trial, the route of administration of the batyl alcohol was changed. The substance was brought into solution by the aid of Tween 80 or 20 (batyl alcohol 2 g., Tween 5 g., in 100 ml. of 1% saline, boiled and cooled rapidly) and given by slow intravenous injection (25–50 ml.) daily, to a further two bullocks at the critical stage of bracken poisoning. These animals had leucocyte counts of 2500 and 2000/mm.^{3} and platelets of 65 000 and 60 000/mm.^{3} respectively. After a course of four daily intravenous injections of batyl alcohol, coupled with a wide-span antibiotic therapy, the leucocytes had risen in both animals to 4000/mm.^{3} and the platelets to 120 000 and 70 000/mm.^{3} respectively. Both animals recovered.

A limited number of field cases of bracken poisoning in cattle have since been successfully treated by this therapy.

We wish to thank Roche Products Ltd. for generously supplying us with DL-batyl alcohol.

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Potassium and Sodium Movements in Sheep Blood Cells. By C. R. B. JOYCE and M. WEATHERALL. (*Department of Pharmacology, London Hospital Medical College, London, E.* 1)

The K and Na contents of the red cells of different sheep vary greatly, within a range of 5–100 m-equiv./l. cells and 15–120 m-equiv./l. cells respectively (Kerr, 1937; Evans, 1954). The rate at which ionic exchanges occur in cells from individuals taken from various points in these ranges has been investigated by incubating whole blood taken from Scottish Blackface sheep in Krebs type saline containing ^{24}Na or ^{42}K for periods up to 6 hr. at 37°. Some cells so treated were transferred after a time to similar media of lower specific activity, so that the efflux of tracer from the cells could be observed.

The cell K and Na remained approximately constant during the experiments. Both ^{24}Na and ^{42}K entered the cells more rapidly at first than later: the decline in rate was greater than would occur with the simple exponential relationship of a two-compartment system. This suggested that the cations exchanged in more than one fraction. In efflux experiments after a short period of influx, the overall specific activity of the cells at first fell to below that of the efflux medium and then rose again, further indicating the presence of a fast fraction of each cation. The rapidly exchanging fraction of the cell K appeared to contain less than 5 m-equiv./l. cells, regardless of the total cell K concentration, and the rapid fraction of the cell Na about 10–20 m-equiv./l. cells, being possibly a little higher but not proportionately larger in cells with a high total Na concentration. The size of the rapid fractions are therefore independent of the total ionic concentration, and the difference between different cell populations is mainly in the relatively fixed part of the cell cation.

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Amphetamine in the Urine of Mental Hospital Patients. By P. H. CONNELL (introduced by R. RODNIGHT). (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

In a study of forty-two cases of psychosis due to amphetamine intoxication the clinical picture was found to be indistinguishable from paranoid schizophrenia. Since amphetamine addiction may also be confused with other psychiatric complaints an objective test to assist in the diagnosis of this condition is clearly required.

Richter (1938) found that amphetamine was excreted in the urine for 48 hr. following oral administration. Analysis of the patients' urine was therefore chosen as a likely procedure. For this purpose the methyl-orange method of Brodie & Udenfriend (1945) for assaying basic drugs in urine was adapted for clinical use. Utena, Ezoe & Kato (1955) have also used this method to investigate the excretion of methyl-amphetamine in three addicts. In the present work, 10 ml. of urine (pH 10) was extracted with 25 ml. of benzene; the benzene phase was then equilibrated with 1 ml. of buffered methyl-orange solution and after centrifuging 20 ml. of it was shaken with 3·5 ml. of N-HCl. The resulting colour in the aqueous phase was measured spectrophotometrically. By this technique six urines could be analysed in less than 2 hr.

Normal urines gave blank values equivalent to a maximum of 0·7 μg. amphetamine/ml.; this was only a small proportion of the amphetamine found in the patients' urines (6–112 μg./ml.). Since other drugs, notably chlorpromazine, may give positive values, paper chromatography was used in a proportion of cases to confirm the presence of amphetamine in the urine.

The urines of ten subjects, admitted to an observation ward as paranoid psychotics, were tested by the methyl-orange method morning and evening for a week. Excretion of amphetamine continued for 4–7 days after the ingestion of the last dose. Reasons will be given for attributing this long continued excretion to the size of the dose and not to any abnormality in amphetamine metabolism. The amounts ingested varied from 128 to 975 mg. of amphetamine (base).

In other experiments considerable amounts of amphetamine were found in the urine of a normal subject after moderate use of an amphetamine inhaler for 24 hr. This result appears to contradict the findings of Simpson & Simon (1937), who concluded that absorption of significant dose by inhalation was impossible.

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A Comparative Study of [16-^{3}H]Progesterone Metabolism in Pregnant and Non-pregnant Women. By W. H. PEARLMAN. (*Guy's Hospital Medical School, London, S.E.* 1)

The percentage recovery of administered progesterone as urinary pregnanediol has been reported to be much higher (about threefold) in pregnancy than in the non-pregnant state in the absence of luteal function (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953). The validity of these observations might bear re-examination in view of the experimental difficulties in determining the additional amount of pregnanediol in pregnancy urine which arises from the administered hormone. These difficulties may be obviated by employing isotopically labelled progesterone. Accordingly, [16-^{3}H]progesterone was prepared (Pearlman, to be published) and injected intramuscularly into three women in late pregnancy and into two oophorectomized-hysterectomized patients who served as a control group. The urinary recovery of tritium in the neutral ketonic and non-ketonic fractions and as pregnane-3α:20α-diol was determined. The percentage recovery of progesterone as urinary pregnane-3α:20α-diol was calculated to be: (*a*) in the pregnant subjects, 6, 15 and 14 %, and (*b*) in the non-pregnant subjects, 27 and 14 %. These findings do not support the earlier observations made without the aid of isotope that the percentage recovery of the metabolite is greatly increased in pregnancy. The isotopic data also permitted a calculation of the endogenous production of progesterone to be of the order of one-quarter of a gram per day in late pregnancy. Of the total amount of tritium injected, 31 % (average value for all subjects) was recovered in the neutral urinary extracts, the isotope being distributed between the ketonic and non-ketonic fractions in a proportion of about 1:4.

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The Metabolism of the Herbicide, *p*-Chlorophenoxyacetic Acid by a Soil Micro-organism—the Formation of a β-Chloromuconic Acid on Ring Fission. By W. C. Evans and P. Moss. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Evans & Smith (1954) isolated from soil a Gram-negative motile organism (pseudomonas type), which grew under aerobic conditions in a mineral salt medium containing *p*-chlorophenoxyacetic acid (up to 0·1%, w/v). 2-Hydroxy-4-chlorophenoxyacetic acid and 4-chlorocatechol were isolated from such cultures, and these compounds obeyed criteria indicating their participation in the metabolic pathway of the initial substrate. Occasionally, a substance having an absorption maximum at 264 mμ. could be detected in cultures, which, after the usual isolation procedures was identical with a synthesized β-chloromuconic acid, m.p. 235–238° (Boeseken & Metz, 1935). Washed cells grown on *p*-chlorophenoxyacetic acid, when incubated with 4-chlorocatechol always led to an accumulation of a β-chloromuconic acid, which was eventually metabolized with the liberation of chloride ions. The cells, however, were incapable of metabolizing the synthetic β-chloromuconic acid, or that obtained from the culture after its isolation. This suggests that cells grown on *p*-chlorophenoxyacetic acid contain an enzyme specific towards another isomer (probably *cis-cis*) of β-chloromuconic acid, actually present in the culture, and inverted to the more stable *cis-trans* form during isolation. The postulated *cis-cis* acid has not yet been obtained synthetically by us.

Infrared examination of the synthetic β-chloromuconic acid, and the product isolated from the culture, suggests that the former is the *cis-trans* isomer, whilst the latter, in addition to containing this, is contaminated with a closely related lactone.

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***In vitro* Resynthesis of Energy-rich Phosphates in Cerebral Tissue.** By J. Thomas. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E. 5*)

In pieces of isolated brain respiring in simple oxygenated glucose salines, appreciable resynthesis of energy-rich phosphates occurs, but the levels maintained remain below those *in vivo* (McIlwain, 1951, 1955). By making more creatine available to slices in saline, it is possible to increase the resynthesis of the energy-rich phosphate of creatine (Thomas, 1956). The effect on nucleotide resynthesis of incubation in saline fortified with the nucleotide precursors, purines and nucleosides is now reported. The purines, adenine, hypoxanthine and guanine, although assimilated by brain slices from saline, have little effect on the normal levels of 0·89 and 0·17 μmoles/g. tissue observed for adenine and guanine nucleotides respectively. With the nucleosides, adenosine and inosine, the levels of adenine nucleotides are 1·27 and 1·13 μmoles/g. tissue respectively, and guanosine increases the guanine nucleotide to 0·21. The highest resynthesis was obtained after 3 hr. incubation in saline containing a 1 mM mixture of creatinine, adenosine and guanosine, the levels of adenine and guanine nucleotides being at 1·56 and 0·27 μmoles/g. tissue respectively.

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DEMONSTRATIONS

Apparatus Designed for Studying Separated Tissues. By P. J. W. Ayres,* H. L. Buddle, P. J. Heald, H. McIlwain and R. Rodnight. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London, S.E.* 5)

Current models of apparatus designed for the following purposes will be demonstrated. They have in the main already been described (McIlwain & Buddle, 1953; Ayres & McIlwain, 1953; Rodnight & McIlwain, 1954; Heald & McIlwain, 1956).

(1) Mechanical chopping of tissues. The instrument makes cuts in fresh tissues at predetermined distances yielding uniform slices or suspensions with minimal disruption of cellular structure.

(2) Application of electrical pulses to separated tissues in aqueous media. This comprises sources of electrical pulses with different voltage-time patterns, and special vessels in which tissues are incubated during the passage of pulses.

(3) Study of tissue fragments with little or no added aqueous phase, and in oils. Manometric apparatus designed for this purpose, and the procedure in carrying out measurements and in studying the action of electrical and other agents stimulating and inhibiting metabolism will be shown.

(4) Maintenance and rapid transfer of tissue sections. A holder will be demonstrated, designed for thin sections of tissue and enabling them to be transferred rapidly to fixing agents after adding reagents or during the passage of electrical pulses. An incubating device and applications of the technique will be shown.

* Present address: Middlesex Hospital, London, W. 1.

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A New Supporting Medium for Zone Electrophoresis. By J. Kohn (introduced by E. J. King). (*Queen Mary's Hospital, Roehampton, London, S.W.* 15)

Filter paper, which is most commonly used as a supporting medium for zone electrophoresis, is not a very satisfactory material. It cannot, for instance, be made completely transparent for scanning purposes. In the search for a more satisfactory medium, bacteriological membrane filters were tried and found to possess properties which appear to make them superior to filter paper. This material is here presented. It is prepared from cellulose acetate; the pores vary in size from 0·5 to 3 μ. The material is brittle when dry but has considerable tensile strength and pliability when wet. The most satisfactory thickness of the strips appears to be of the order of 0·007 in. As the material is comparatively thin and the pores fine, it is recommended that strips of lengths shorter than usual, e.g. 18–20 cm., be used, in order to prevent excessive evaporation in the centre part. To fit these shorter strips into the commonly used tanks, suitable lengths of filter paper can be made to connect the electrophoretic strip proper with the buffer solution. The electrophoresis is performed in the same way as in the filter-paper method, the same apparatus and buffers being used. After the run is finished, the strip is dried at approx. 90° in a hot-air oven for a period of 15 min. or more, if so desired. It is then stained in the usual way, washed and dried. Immersion in a clearing liquid of suitable refractive index renders the strip completely transparent and eminently suitable for scanning; the interfering factor of semi-transluscent fibres of varying thickness and distributions is eliminated. The grain of the stained bands is very fine and the separation of the fraction is usually very neat. Another property of the material here presented is its solubility in certain organic solvents. This offers some very interesting possibilities, as the bands containing the various fractions can be cut out and completely dissolved, leaving all the fractionated substance in the solution, with or without a stain. The strips can also be embedded in a suitable medium making them transparent, dry and firm, like celluloid. Work on this technique is still proceeding. The membrane filters originally used are manufactured by Courtaulds Ltd. and distributed by Messrs. Oxo Ltd. The final product was kindly prepared to my specification by Courtaulds and it is hoped that they will become commercially obtainable through Messrs Oxo Ltd. (Medical Department).

A Simplified Cryostatic Drier for Histopathological Study of Brain Enzymes. By D. NAIDOO and O. E. PRATT. (*Department of Neuropathology, Institute of Psychiatry, University of London, Maudsley Hospital, London, S.E.* 5)

This apparatus was developed to preserve the histological integrity of tissue at the same time as retaining a high level of enzyme activity. Several grams of vitrified brain tissue can be dried simultaneously, without employing diffusion pumps or auxiliary heaters.

Frozen brain slices 1–2 mm. thick are dried in six tubes arranged radially around a P_2O_5 moisture trap mounted on a two-stage rotary vacuum pump. Each tube is immersed in a vacuum flask containing a mixture of liquid and solid ethyl phenyl ether (phenetole) maintained at a temperature of $-34°$ by adding solid CO_2 periodically.

Each drying tube has a detachable Pirani pressure gauge head and can be isolated from the drier by a diaphragm valve. The dryness of the tissue is estimated from the rate at which the pressure rises over the tissue after closing the isolation valve. It is found that drying is adequate when the reading has remained below a level of 50 μm Hg./min./g. fresh tissue for 12 hr.

Each tube may be operated independently and contains paraffin wax so that the tissue is infiltrated under vacuum when dry without contact with the air.

The Localization of Hydrolytic Enzymes in Normal and Pathological Brain Tissue. By D. NAIDOO and O. E. PRATT. (*Department of Neuropathology, Institute of Psychiatry, University of London, Maudsley Hospital, London, S.E.* 5)

The problem of the preparation of brain tissue for the microscopic study of enzymes in their normal structural relationship requires the application of new methods. Chemical fixation procedures suffer from the disadvantages that soluble enzymes and cofactors may be lost or displaced, and that many enzymes may be inactivated. The histochemical techniques developed by Gomori for alkaline and acid phosphatases have been widely applied, but the pictures obtained with a large variety of substrates are indistinguishable and only a fraction of the activity of the more stable enzymes survives fixation embedding (Gomori, 1952). A major problem is to extend the field of study beyond these two rather stable enzymes. One way is to use tissue sections prepared by the freeze-drying technique described in the preceding abstract and to de-wax by treatment with light petroleum. It has been possible to localize the specific phosphatases, 5′-nucleotidase, thiamine pyrophosphatase and Mg- and Ca-activated adenosinetriphosphatases, as well as the non-specific acid and alkaline phosphatases (Naidoo & Pratt, 1953, 1956). The activity of these enzymes is of a similar order to that found in saline suspension of fresh tissue (Pratt, 1953) and the conditions under which activity appears are consistent with the known properties of the enzymes.

However, some enzymes may be too soluble to be localized (e.g. inorganic pyrophosphatase) or may not survive drying (e.g. cholinesterases). It may be possible to study such enzymes in frozen sections cut from unfixed tissue.

An increase in 5′-nucleotidase activity in the rat brain during maturation has been investigated (Naidoo & Pratt, 1954). The histological and biochemical results to be demonstrated are complementary and suggest that this enzyme is closely associated with myelination of nerve fibres. This method, which takes account of both biochemical and histological factors, is proving useful in its application to pathological problems.

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The Estimation of Ascorbic Acid and its Oxidized Forms in Brain and Cerebrospinal Fluid.

By G. H. SLOANE-STANLEY. (*Department of Biochemistry, Institute of Psychiatry, University of London, Maudsley Hospital, London, S.E.* 5)

This method has been developed from that of Roe, Mills, Oesterling & Damron (1948), which was not found entirely satisfactory as described. Everything is done at 0–4° unless stated otherwise.

To 1 g. of ground brain (diluted with 15 ml. water) or 16 ml. of cerebrospinal fluid, 4 ml. of 2·5 % (w/v) $SnCl_2.2H_2O$ in 5 % (w/v) HPO_3 (British Drug Houses Ltd.) is added and the whole centrifuged. One-third of the extract (Fraction I) is mixed with 0·25 vol. of 0·5 % (w/v) thiourea in 1 % (w/v) HPO_3, to stabilize ascorbic acid. For the reduction of ascorbone, Fraction II (6·5 ml.) is mixed with 0·105 ml. of potassium citrate solution (saturated at room temperature), and gassed with H_2S for 2·5 min. (pH now approx. 3·5). The tube is then stoppered and put into a 37° bath for 2·25 min., cooled in ice, given 3 drops of conc. HCl and centrifuged. The supernatant is mixed with 0·25 vol. of 5 % (w/v) thiourea in 1 % (w/v) HPO_3. The rest of the extract (Fraction III) is made yellow with Br_2 to oxidize the ascorbic acid, then gassed with N_2 until free of Br_2, and mixed with 0·25 vol. of 5 % (w/v) thiourea in 1 % (w/v) HPO_3. Standards are prepared according to Roe *et al.* (1948). Two 2 ml. samples of each fraction are then mixed with 0·5 ml. each of 2 % (w/v) 2:4-dinitrophenylhydrazine in '9N-H_2SO_4' (Roe & Kuether, 1943), while a third (blank) sample receives '9N-H_2SO_4' alone. All samples are then incubated at 30° for 15 hr. They then receive '85 % H_2SO_4' and are left to stand and their densities are read as described by Roe & Kuether (1943), except that 2 ml. of acid instead of 2·5 ml. is added to each 'blank' and followed by 0·5 ml. of 2 % (w/v) dinitrophenylhydrazine in '85 % H_2SO_4', and that the final solutions are centrifuged if cloudy.

Recoveries have averaged: in Fraction I, ascorbic acid 0, ascorbone and diketogulonic acid 94 %; in Fraction II, ascorbic acid and ascorbone 0, diketogulonic acid 89 %; in Fraction III, all three substances 99 %.

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Estimation of Primary Arylalkylamines by Salicylaldehyde. By A. MCCOUBREY. (*Department of Biochemistry, Institute of Psychiatry, University of London, Maudsley Hospital, London, S.E.* 5)

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **357th Meeting** *of the Biochemical Society was held in the Department of Biochemistry of St Mary's Hospital Medical School on Saturday, 15 December, at 10.45 a.m., when the following papers were read:*

COMMUNICATIONS

The Distribution of the Volatile Fatty Acids formed from Dried Grass by the Action of Rumen Liquor in the Artificial Rumen.

By A. J. G. Barnett and R. L. Reid. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

In a previous study, Barnett & Reid (1955, 1956) have indicated the general nature, amounts and distribution of the volatile fatty acids produced in the artificial rumen from thirteen samples of fresh grass, obtained from the same ley, over two growing seasons. The study has now been extended to the corresponding dried grass samples and each of these has been examined, first as an entity and secondly, from the point of view of its water-soluble and water-insoluble fractions, which are referred to as grass extract and extracted grass respectively. In fresh grass, acetic acid is produced in major amount early in the year while later on, propionic acid is produced in greatest quantity. With dried grass, acetic acid is the preponderating acid at all times. Grass extract produces an abundance of acetic acid, but in the case of extracted grass, the amounts of acetic and propionic acids are very similar and do not vary throughout the season. The total acid production declines with maturity of the grass but the proportions of acetic, propionic, butyric and valeric acids in the dried grass and in its separates do not show seasonal variation to any degree. A study of the volatile fatty acids derivable from dried grass and from its separates reveals that the former exists, as would be expected, as something of a composite of the latter. The acids are produced with great rapidity from the grass extract, but much more slowly in the case of extracted grass which tends to resemble cellulose to some extent in its behaviour in the artificial rumen.

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The Interaction of Insulin Monolayers with Polysilicic Acid.

By P. F. Holt and C. W. Went. (*University of Reading*)

Substances which react with proteins may markedly alter the properties of a protein monolayer. The interaction of silicic acid with monolayers of gliadin (Schulman & Rideal, 1937), methaemoglobin (Holt & Bowcott, 1954), and collagen (Holt & Clarke, 1955), has been studied by the monolayer technique.

The force/area (f/a) characteristics of insulin films on saline subsolutions have been compared with those of films on saline and polymerized silicic acid over a wide pH range. Films on saline give f/a curves of the usual S-shape, collapsing at about 20 dynes/cm. An area (at 10 dynes/cm.)/pH plot is W-shaped (maximum about pH 5·5; minima at pH 3·5 and 8). Except at pH 5·4 to 6·1 the f/a curves of films on 0·025 M silicic acid are similar to those on silica-free subsolutions, but the areas are slightly larger between pH 3 and 9.

Between pH 5·4 and 6·1 the f/a curves of films are coincident at very low pressures (<2 dynes/cm.) with those relating to silica-free subsolutions. At higher pressures the f/a curves rise more steeply; the films are much less compressible and do not collapse even at pressures >30 dynes/cm. On 0·005 M silicic acid similar but smaller effects are observed; at pH 5·4–6·1 the f/a curves remain coincident with those relating to silica-free subsolutions up to pressures of 6 dynes/cm., but beyond this, rise more steeply and are parallel with those obtained on 0·025 M silicic acid.

Apparently silicic acid reacts with insulin between pH 3 and 9, the adsorbed polymers preventing close packing of the protein chains on compression. Between pH 5·4 and 6·1 a rigid polymerized network forms beneath the film, considerably altering its properties. Silicic acid adsorbed in concentrations too dilute for polymerization to occur may polymerize when the two-dimensional concentration is increased by compressing the film. Insulin

behaves somewhat differently from collagen; this is correlated with their structures.

A grant to one of us (C.W.W.) from the British Rayon Research Association, and gifts of pure insulin from the Wellcome Foundation, are gratefully acknowledged.

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Oxidation by Ultra-sonic Extracts of *Acetobacter suboxydans*. By J. A. FEWSTER.* (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Washed organisms of *Acetobacter suboxydans* (A.T.C.C. 621) grown on media containing glucose and chalk (in suspension) or mannitol oxidize glucose, gluconic acid and 5-oxogluconic acid extensively (Fewster, 1956). The organisms have now been disintegrated with a Mullard ultrasonic oscillator and the oxidation by the resulting extracts of a number of substrates at 30° in phosphate buffer pH 6·0 studied manometrically. Glucose and gluconic acid are oxidized to 2- and 5-oxogluconic acid, identified chromatographically; the oxo acids are not attacked. The extent of oxidation of glucose is not increased by addition of ATP and Mg^{2+}, DPN, or cytochrome *c*, and the rate is not appreciably decreased by 10^{-3}M dinitrophenol or by the absence of added inorganic phosphate; 10^{-2}M dinitrophenol or a rise in pH to 8·0 prevents the oxidation of gluconic acid. With the following aldoses O_2 consumption is 0·5 mole/mole, corresponding to the formation of aldonic acids: 3- and 6-*O*-methyl-D-glucose, D-mannose, D-galactose, D-xylose, D-lyxose, L-arabinose. D-Ribose, D-arabinose and L-xylose are oxidized very slowly. Glucose 6-phosphate, which is not attacked by the washed organisms (Fewster, 1956), slowly consumes 0·5 mole O_2/mole as do fructose 6-phosphate and fructose 1:6-diphosphate.

Glycerol, *meso*-erythritol, D-arabitol, D-mannitol and D-sorbitol are oxidized to dihydroxyacetone, L-erythrulose, D-xylulose, D-fructose and L-sorbose, respectively; these ketoses and dulcitol are not attacked. Lactate, pyruvate, ethanol and acetaldehyde are oxidized to acetate. An extract obtained from *A. suboxydans* by alumina grinding (King & Cheldelin, 1954) possessed the ability to oxidize dihydroxyacetone; glucose, pyruvate, ethanol and acetaldehyde were acted upon in a manner similar to that of the extract reported here.

Centrifuging the extract at 25 000 *g* yields a particulate fraction, rich in cytochrome, which oxidizes glucose to the extent of 1 mole and glycerol and mannitol to 0·5 mole O_2/mole substrate; addition of DPN increases the rate. The supernate of this fraction, essentially free of cytochrome after centrifuging at 100 000 *g*, slowly oxidizes glucose to gluconic acid but does not attack glycerol or mannitol. Arcus & Edson (1954) report the polyol dehydrogenase activity of fractionated extracts of *A. suboxydans*; Widmer, King & Cheldelin (1956) describe a particulate fraction which oxidized glucose and a number of polyols to the extent of 0·5 mole O_2/mole.

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* Guinness Research Fellow in Microbiological Biochemistry.

The Metabolism of [^{14}C]Hexachloroethane. By W. R. JONDORF, D. V. PARKE and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

Hexachloroethane (C_2Cl_6) is used in veterinary medicine as an anthelminthic. It is a highly volatile solid, m.p. 187° (sealed tube). C_2Cl_6 containing ^{14}C in both carbon atoms and free from other chlorinated ethanes was prepared from barium carbide, and fed to rabbits in doses of 0·5 g./kg. It is very slowly metabolized and only 5 % of the radioactivity appears in the urine in 3 days. During this time, however, 14–24 % of the radioactivity appears in the expired air. The rest of the material appears to be in the tissues and intestinal tract but at present we have no exact measurement of this, owing to the difficulty of counting highly volatile compounds with the apparatus available.

An examination of the urinary metabolites showed that C_2Cl_6 had undergone some amazing transformations. The following compounds were identified by chromatographic and isotope-dilution techniques: trichloroethanol (1·3%), dichloroethanol (0·4%), trichloroacetic acid (1·3%), dichloroacetic acid (0·8%), monochloroacetic acid (0·7%) and oxalic acid (0·1%) (the figures given are averages for three experiments). Monochloroethanol, acetate, formate, bicarbonate and urea were not formed from C_2Cl_6.

In the expired air, there was found CO_2, C_2Cl_6, tetrachloroethylene and 1:1:2:2-tetrachloroethane. Trichloroethylene (CCl_2:CHCl) was not found.

Of the urinary metabolites, monochloroacetic acid is a highly toxic substance.

This work was supported by a grant from the Agricultural Research Council.

The Fate of [^{14}C]Phenylhydrazine in the Rabbit. By W. M. McIsaac, D. V. Parke and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Phenylhydrazine labelled with ^{14}C was synthesized and administered orally to rabbits in doses of 50 mg./kg. After 48 hr. some 30–50% of the radioactivity appeared in the urine and a further 5–15% was excreted in the next 48 hr. Radioactive metabolites, however, were still being excreted 10 days after dosing. In animals killed 4 days after dosing, 5–10% of the dose of ^{14}C was found in the blood, almost entirely in the erythrocytes. The spleen contained 1%, and the stomach contents 3–5% of the dose.

Paper chromatography of the urine revealed three radioactive metabolites, a glucuronide (R_F 0·3 in *iso*propyl ether/water), pyruvic acid phenylhydrazone (R_F 0·86) and α-oxoglutaric acid phenylhydrazone (R_F 0·95). After acid hydrolysis of the urine, the presence of *p*-hydroxyphenylhydrazine and phenylhydrazine was indicated. Conjugated glucuronic acid and ethereal sulphate amounted to 40% and $\nearrow$2% of the dose respectively. Isotope dilution studies showed that in 4 days after dosing, some 30% of the dose was excreted as conjugated *p*-hydroxyphenylhydrazine, and 6 and 4% as the phenylhydrazones of pyruvic and α-oxoglutaric acids, respectively. Preliminary observations suggest that pyruvic acid phenylhydrazone is less than one-half as toxic as phenylhydrazine.

Acetylphenylhydrazine, *o*-, *m*- and *p*-aminophenols, phenol, quinol, glucose phenylhydrazone and glucosazone were not found as urinary metabolites. The presence of pyridoxal phenylhydrazone, however, is suspected.

Substrates for *N*-Acetyl-β-glucosaminidase. By J. Borooah, D. H. Leaback and P. G. Walker. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

The hydrolysis of ethyl, *p*-nitrophenyl and *o*-nitrophenyl *N*-acetyl-β-glucosaminides by β-glucosaminidase has been investigated. The enzyme from ram testis was partially purified by fractionation with ammonium sulphate. The substrates were prepared from 1-chlorotetra-acetylglucosamine by a general method for the synthesis of alkyl and aryl *N*-acetyl-β-glucosaminides (Leaback & Walker, 1956). Enzyme activity was measured by the liberation of ethanol, *p*-nitrophenol or *o*-nitrophenol which were estimated spectrophotometrically, ethanol by the reduction of diphosphopyridine nucleotide in the presence of alcohol dehydrogenase (Bücher & Redetzki, 1951; Kuhn & Tiedemann, 1954), and the nitrophenols by the absorption maximum of the anionic form. In no case was deproteinization of the reaction mixture necessary.

The optimum pH for hydrolysis of ethyl *N*-acetyl-β-glucosaminide in 0·05M citrate buffer was 4·4 and the Michaelis constant (K_m) was 4·9 mM. The corresponding values for *p*-nitrophenyl and *o*-nitrophenyl *N*-acetyl-β-glucosaminides were pH 4·4 and 1·4 mM and pH 4·8 and 2·3 mM respectively. The relative maximum rates of hydrolysis of the ethyl, phenyl, *o*-nitrophenyl and *p*-nitrophenyl substrates were in the ratio 1:10:20:25. The hydrolysis of phenyl *N*-acetyl-β-glucosaminide was inhibited competitively in the presence of ethyl *N*-acetyl-β-glucosaminide (K_i 4·5 mM). The similarity in the values of K_i and K_m for this compound makes it probable that the same enzyme is responsible for the hydrolysis of alkyl and aryl *N*-acetyl-β-glucosaminides.

A sensitive and convenient method for the assay

of β-glucosaminidase using *p*-nitrophenyl *N*-acetyl-β-glucosaminide as substrate has been applied to measurement of the hitherto undetected activity of the enzyme in serum.

We wish to thank Messrs Benger Ltd., Holmes Chapel, Cheshire, for generous supplies of freeze-dried testis extract.

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N-Acetyl-β-glucosaminidase in Rat Tissue Preparations. By Doreen Pugh, D. H. Leaback and P. G. Walker. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

The activity and distribution of β-glucosaminidase in homogenates (1 %, w/v) of rat tissues prepared in water, 0·25 M sucrose and 0·14 M-NaCl has been studied using the technique of centrifugal fractionation. In a saline homogenate of rat kidney about 60 % of the enzyme was sedimentable at 25 000 *g* and is presumably associated with subcellular particles. After disintegration of the particles in the Waring Blendor the total activity of the preparation was unchanged but none of the enzyme was sedimentable (cf. Walker, 1952). The β-glucosaminidase activity of tissue homogenized in water was 20 % lower than that of the same tissue homogenized in isotonic saline. About 40 % of the enzyme in a water homogenate was sedimentable but this fraction was decreased only slightly by treating the homogenate in the Waring Blendor. The distribution was not, therefore, dependent on the integrity of subcellular particles. Making a water homogenate 0·14 M with respect to NaCl, KCl or Na_2SO_4 increased its β-glucosaminidase activity by 20 % and reduced the sedimentable fraction of the enzyme to about 10 % of the total. After dialysis of the homogenate, on the other hand, the total activity was slightly reduced and all of the enzyme was sedimentable. Alterations in the activity and distribution of β-glucosaminidase produced by changes in electrolyte concentration were completely reversible. Qualitatively similar effects were observed with homogenates prepared in 0·25 M sucrose. The precipitation of β-glucosaminidase which occurs at low salt concentrations results in partial, reversible, inactivation of the enzyme and it seems that, in order to avoid artifacts, measurements of the activity and distribution of the enzyme in dilute tissue preparations should be carried out in the presence of adequate concentrations of electrolyte. The importance of electrolyte concentration in determining the protein content of the 'soluble' fraction of homogenates of rat liver has recently been pointed out by Anderson (1956).

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Inhibition and Activation of β-Glucuronidase in Urine. By M. A. M. Abul-Fadl. (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London, S.W. 3*)

The inhibition of β-glucuronidase by normal tissue constituents and body fluids is well known (Fishman, Altman & Springer, 1948; Becker & Friedenwald, 1949; Fishman, 1952; Walker & Levvy, 1953). Dialysis of human urine caused variable increases in β-glucuronidase activity. Dilution of urine sometimes caused a further increase in activity beyond that obtained by dialysis, indicating the presence of dialysable and non-dialysable inhibitors. The dialysable inhibitor in untreated urine is stable in hot acid solution but labile to alkali. From 5 to 10 % of the inhibitory activity could be extracted with butanol, while most of the remainder could not be extracted by solvents, except after acid hydrolysis. After evaporating the urine to dryness under vacuum the inhibitory substances were extracted by pyridine or dioxan. Experiments by paper chromatography indicate that the inhibitory substances present in solvent extracts are an organic acid present in different forms of combination, one of which (in the dioxan extract) is possibly a carbohydrate conjugate.

A 50 % inhibition of β-glucuronidase preparations from different sources was produced by 0·075 mg. of the purified material per ml. enzyme–buffer–substrate mixture. It is acid in reaction about 400 m-equiv./l., thermostable at the normal pH of urine but sensitive to acid and alkali treatment in the cold. Inhibition varied with substrate and inhibitor concentration.

Over seventy known urinary constituents or related compounds were found not to inhibit β-glucuronidase activity when present in concentrations up to twice those at which they are normally present in urine. 1:4-Saccharolactone, saccharic acid, glycyrrhizic acid, rutin, mesotartaric, mucic, malic, taurocholic, glycocholic, tartronic, cholic, deoxycholic, citric, oxalacetic, fumaric, ascorbic, maleic, succinic, lactic, pyruvic and salicylic acids are inhibitors of urinary β-glucuronidase in decreasing order of activity.

Protamine, crystalline bovine serum albumin, DNA, gelatin, chymotrypsin, spermine and yeast ribonucleic acid are among the substances known to enhance the β-glucuronidase by restoring activity, which is considerably reduced on dilution of highly purified preparations from calf spleen and liver (Bernfeld, Bernfeld, Neisselbaum & Fishman, 1954). Ethylene glycol has also been shown to increase the activity of β-glucuronidase in cow adrenal (Nayyar & Glick, 1956). Only protamine, and crystalline egg albumin activated the β-glucuronidase of some urines. Protamine caused a considerable increase (1·3- to 20-fold) in the activity of all dialysed or diluted urines, the activation being greatest at pH 3·8.

Plasma β-glucuronidase was not affected by protamine between pH 3·5 and 5·5 before or after dialysis. Enzyme preparations from tissues, sometimes showed slight activation with protamine at pH 4·5.

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Deoxyribonucleic Acid Synthesis in Bacteriophage-infected *Escherichia coli*. By L. V. Crawford (introduced by K. McQuillen). (*Department of Biochemistry, University of Cambridge*)

Deoxyribonucleic acid (DNA) synthesis in *Escherichia coli* infected with phage T2 is prevented if protein synthesis is prevented. However, if protein synthesis is allowed to occur for only a few minutes and then stopped, the infected cells can form phage DNA (Cohen, 1947; Burton, 1955; Hershey, Burgi, Garen & Melechen, 1955; Hershey, 1956; Tomizawa & Sunakawa, 1956).

A survey has now been made of an extended range of organisms: *E. coli* B with phages T1–T7 and 201S, and *E. coli* 15T⁻ with a phage, C1, isolated from sewage. The bacteria were infected in a N-free medium and transferred after 10 min. to a peptone-yeast extract medium. Phage adsorption, and probably injection of phage DNA into the bacteria, occurred in the N-free medium under the conditions used. Chloramphenicol (20 μg./ml.) reduced incorporation of $^{35}SO_4$ and [^{14}C]leucine to 1–2 % and reduced the increase of hot 5 % trichloroacetic acid–insoluble N (protein and cell wall) to less than 9 %. This indicates that protein synthesis was reduced to a very low level.

As other authors have found for T2, inhibition of protein synthesis in cells infected with T2, T4 and T6 prevented DNA synthesis. However, with phages T1, T3, T5, T7, 201S and C1, considerable increases of DNA occurred whether chloramphenicol was added at the time of transfer to growth medium, or 5 min. earlier to the N-free medium. Thus prior protein synthesis was necessary for the synthesis of the DNA of the T-even phages but not, apparently, for the other phages tested.

This difference could be connected with the occurrence of the pyrimidine 5-hydroxymethylcytosine only in the T-even phages. The metabolism of this compound might involve the formation of new enzymes. Since the DNA's of the other phages do not contain 5-hydroxymethylcytosine it is difficult to be certain that phage DNA was formed in the presence of chloramphenicol but for several reasons it seems likely. If this is so, the generalization that the formation of a new DNA requires prior protein synthesis is untenable.

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A Study of the Mechanism of Penicillin Biosynthesis using [α−^{3}H]- or [β−^{3}H]Cystine. By H. R. V. Arnstein. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7) and J. C. Crawhall. (*Department of Biochemistry and Chemistry, The Medical College of St Bartholomew's Hospital, London, E.C.* 1)

Previous work has established that L-cysteine is a precursor of the sulphur, the side-chain nitrogen and the β-lactam carbon atoms of penicillin (Arnstein & Grant, 1954*b*), whilst the carbon atoms of the penicillamine moiety are derived from valine or from a close derivative of this amino acid (Stevens, Vohra & DeLong, 1954; Arnstein & Grant, 1954*a*; Arnstein & Clubb, 1957). Possible mechanisms by which cysteine and valine might condense to give the fused β-lactam-thiazolidine ring structure of penicillin have now been investigated with DL-cystine labelled with tritium in either the α- or the β-position.

DL-[α-^{3}H]Cystine was synthesized by decarboxylating benzylthiomethylacetamidomalonic acid, or its mono ethyl ester, in the presence of ^3H_2O, followed by hydrolysis of the product with HCl and removal of the *S*-benzyl group. The preparation of DL-[β-^{3}H]cystine involved the condensation of C^3H_2O with ethyl *N*-dithiobenzyloxycarbonamidomalonate, cyclization of the product to the thiazoline and hydrolysis, essentially as described by Crawhall & Elliott (1951).

Experiments with washed mycelium of *Penicillium chrysogenum* WIS 51–20 (Halliday & Arnstein, 1956) showed that the conversion of DL-[α-^{3}H]-cystine into penicillin was much less than that of DL-[β-^{14}C]cystine, the dilution of molar radioactivity being 20·2 and 9·1, respectively. The less efficient utilization of [α-^{3}H]cystine is probably due to an isotope effect, since in a full fermentation the incorporation of [α-^{3}H]cystine into the mycelial protein was also less than that of [β-^{14}C]cystine, although the relative incorporation into penicillin and protein was similar in both cases.

Degradation experiments showed that penicillin biosynthesized from [α-^{3}H]cystine was labelled almost exclusively at position 6 of the β-lactam ring. In a similar experiment with [β-^{3}H]cystine, penicillin contained significant radioactivity which was present almost entirely in the penilloaldehyde moiety, presumably at position 5.

These experiments show that penicillin biosynthesis does not involve the intermediary formation of an αβ-dehydrocysteine derivative, but that the β-lactam ring may be formed by an oxidative condensation between the β-position of cysteine and the peptide nitrogen of a derivative such as cysteinylvaline.

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α-Mannosidase in Mammalian Tissues. By J. Conchie, June Findlay and G. A. Levvy. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

α-Mannosidase activity has been found to be widely distributed in the tissues of the mouse and the rat. The activity of a given organ was usually similar in the two species. Figures for adult kidney, liver and spleen were about 2500, 900 and 1000 M.U. (mannosidase units) per g. moist tissue respectively, where 1 M.U. liberates 1 μg. *p*-nitrophenol in 1 hr. from 0·002 M-*p*-nitrophenyl-α-mannoside at 37° and pH 4·6. In infants, the figures for these organs were approximately doubled. The activity in skeletal muscle and heart was negligible. Whilst we have no information regarding the natural substrate for this enzyme, mannose is a common constituent of mucoproteins, e.g. ovomucoid (Gottschalk & Ada, 1956) and urinary mucoprotein (Anderson & Maclagan, 1955).

Epididymis was the only organ with a higher activity than kidney, giving figures of 13 000 to 30 000 M.U. per g. tissue in adult mice and rats. In infants and in castrate adults, this figure fell to about 2000 M.U. per g. tissue. Attempts to restore this figure to the normal adult value by injecting castrates with testosterone are in progress, so far without success.

Ovariectomy caused a fall in adult mouse uterine α-mannosidase activity from 1000 M.U. per g. tissue to vanishingly small proportions: the activity was restored to normal by oestrone administration. Injection of adult male mice with menthol or with oestrone approximately doubled liver α-mannosidase activity. In these respects, α-mannosidase in uterus and liver resembles β-

glucuronidase (for reviews, see Fishman, 1955; Levvy, 1956). Epididymis β-glucuronidase activity was not, however, found to be particularly high, nor did it vary markedly with age.

The only human tissues so far studied for α-mannosidase activity are a carcinoma of the vulva, with an activity of 715 M.U. per g., and vaginal fluid, which rose from almost zero in healthy women to as high as 1350 M.U. per g. in cases of uterine cancer.

Like β-glucuronidase, β-*N*-acetylglucosaminidase has been shown to act on degradation products from hyaluronic acid (Linker, Meyer & Weissmann, 1955). A few experiments have been done with the latter enzyme. Adult rat epididymis had a higher β-*N*-acetylglucosaminidase activity than any other tissue studied. This figure was reduced about 15-fold in infant animals. The figure for uterine β-*N*-acetylglucosaminidase activity in mice was halved on ovariectomy and restored to normal on oestrone administration.

One of us (J.F.) is in receipt of a grant from the Medical Research Council. We are indebted to Dr J. Lawson for clinical specimens.

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Ornithuric Acid Formation in Reptiles. By J. N. SMITH. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

It has been reported that frogs, and a tortoise (Komori, Sendju, Sagara & Takamatsu, 1926) can synthesize hippuric acids from ingested aromatic acids, and hippuric acid has been reported as a normal constituent of *Testudo tabulata* urine (Schiff, 1859).

Benzoic acid or *p*-aminobenzoic acid was fed to two species of lizard (*Lacerta viridis*, *Anguis fragilis*), a snake (*Natrix natrix*), two species of tortoise (*Testudo graeca*, *Emys europaeus*) and a crocodile (*Caiman* sp.), and the excreta examined for conjugated metabolites. In all the above species the presence of ornithuric acid or di-*p*-aminobenzoylornithine was detected by paper chromatography. After feeding benzoic acid to *Testudo graeca* small amounts of L-(+)-ornithuric acid, m.p. 183°, $[\alpha]_D^{25}$ 9·7° were isolated from the excreta. Ornithuric acid, m.p. 183°, was similarly isolated from *Emys europaeus* excreta.

In each of the species examined, the ornithuric acid or di-*p*-aminobenzoylornithine was accompanied by much smaller amounts of the corresponding glycine conjugate and by other conjugated metabolites.

The di-*p*-aminobenzoylornithine present in the excreta of *Lacerta viridis* after giving a 0·5 mg./g. dose of *p*-aminobenzoic acid was determined after separation on paper chromatograms. About 20–30% of the dose was excreted as di-*p*-aminobenzoylornithine and 5–10% as unchanged material in the course of the 2 weeks following dosage.

These results show the metabolism of aromatic acids in lizards to be similar to that in birds where the formation of ornithuric acids is well-known. Similar observations have been made recently on chicks fed nicotinamide where, in addition to dinicotinoylornithine and nicotinuric acid, α- and δ-nicotinoylornithines have been detected (Chang & Johnson, 1956).

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The Metabolism of Phenylethylene and Phenylacetylene. By A. M. EL MASRY, J. N. SMITH and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

Ethylbenzene ($PhCH_2CH_3$) is converted in the rabbit mainly to the glucuronide of methylphenylcarbinol and to hippuric acid, with some phenaceturic acid and small amounts of mandelic acid (Smith, Smithies & Williams, 1954; El Masry, Smith & Williams, 1956). The unsaturated derivatives, $PhCH{:}CH_2$ and $PhC{:}CH$ behave differently.

Phenylacetylene (oral dose, 0·4 g./kg.) is relatively stable *in vivo* and much of the dose is exhaled unchanged over a long period. This amounted to 25–37% of the dose in 72 hr. and phenylacetylene

was still being expired for several days after this time. The only urinary metabolite found was phenaceturic acid (10–15% of the dose during 3 days). There was no significant increase in hippuric acid, *p*-hydroxyphenylacetic acid, glucuronides or ethereal sulphates. Traces of mandelic acid were detected in chloroform extracts of the urine. The main reaction was thus

$$PhC{:}CH \rightarrow PhCH_2COOH.$$

In contrast, styrene (0·5 g./kg.) yielded hippuric acid (40%), a glucuronide (6%) and mandelic acid (3% isolated). There was no phenaceturic acid. The glucuronide was isolated as a *tetraacetyl methyl ester* of a *monoglucuronide* of *phenylglycol* (m.p. 140°; $[\alpha]_D^{25}$ 1° ± 2° in $CHCl_3$; Found: C, 55·9; H, 5·7; OMe, 6·1. $C_{23}H_{28}O_{12}$ requires C, 55·7; H, 5·7; OMe, 6·3%). The same glucuronide was isolated when rabbits were fed (±) phenylglycol. This glycol also yielded large amounts of mandelic acid and small amounts of hippuric acid. The probable metabolic reactions of styrene are thus

$$Ph.CH{:}CH_2 \rightarrow \begin{matrix} Ph.COOH \\ \uparrow \\ Ph.CHOH.CH_2OH \end{matrix} \rightarrow Ph.CHOH.COOH.$$

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The Incorporation of [^{35}S]Methionine into Protein Fractions of the Rat Brain. By D. E. Clouet and D. Richter. (*Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff*)

^{35}S-Methionine is rapidly taken up from the cerebrospinal fluid and incorporated into the proteins of the brain (Gaitonde & Richter, 1956). The rate of incorporation has now been determined in nuclear, mitochondrial, microsomal and supernatant fractions separated by differential centrifugation.

Thirty rats of 37 g. mean weight and 28 days mean age were injected intracisternally with DL[^{35}S]-methionine (100 μc./100 g. body weight). Groups of six rats were killed by decapitation at 0·5, 1, 2, 3 and 6 hr. after injection. Brain homogenates in 0·25M sucrose were immediately prepared and separated into particulate and supernatant fractions (Hogeboom & Schneider, 1950). The proteins of the particulate fractions were each separated further by successive extractions with M-NaCl (subfraction A) and N-NaOH (subfraction B) into two soluble and one residual insoluble fraction (subfraction C). The proteins were precipitated with trichloroacetic acid, followed by heat coagulation, and extracted successively with chloroform:ethanol (1:1, v/v), ether and acetone to remove the lipids. The nitrogen, nucleic acid and associated lipid content of each protein fraction were measured, as well as the specific radioactivity of the protein S.

The most rapid uptake of ^{35}S-methionine was found in the microsomal fraction: the specific radioactivity of the protein S in the microsomes at 3 hr. was 3·8 times that of the whole homogenate. The protein which had the highest incorporation rate was a nucleoprotein obtained from the microsomal fraction by further extraction with N-NaOH (subfraction B): this had a specific radioactivity at 3 hr. 4·5 times that of the whole homogenate. It contained 1·9 g. nucleic acid per 100 g. protein.

Determinations carried out at 6 hr. after the injection of ^{35}S-methionine gave evidence of a subsequent fall in the specific radioactivity of the microsomal nucleoprotein, accompanied by a rise in the specific radioactivity of the other fractions. This suggests that the microsomal nucleoprotein may be concerned in the initial amino acid incorporation and transfer of amino acids to other proteins. These observations may be compared with those described for other tissues (Askonas, Simkin & Work, 1956; Hoagland, 1955; Littlefield, Keller, Gross & Zamecnik, 1955).

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6-*O*-Acetylglucopyranose—A Metabolite of *Bacillus megaterium*. By R. B. Duff, D. M. Webley and V. C. Farmer (introduced by J. S. D. Bacon). (*Macaulay Institute for Soil Research, Aberdeen*)

The strain used (N.C.I.B. 8508) was originally isolated from soil and gives high yields of cobalamin (Garibaldi, Ijichi, Snell & Lewis, 1953).

The new metabolite was first observed on a paper chromatogram of a filtrate from the organism growing on a glucose-mineral salts medium. It had $R_{(Tetramethylglucose)}$ 0·28 in butanol-water at 30° and appeared to be a carbohydrate. Separation from mineral salts and excess substrate was effected by ion-exchange resins and chromatography on cellulose respectively and the *substance* obtained crystalline with m.p. 133°; $[\alpha]_D^{20} + 48°$ at equilibrium (*c*, 4·0 in H_2O). (Found: C, 43·1; H, 6·3. $C_8H_{14}O_7$ requires C, 43·2; H, 6·4 %.)

Infrared analysis showed that the substance was an acetylated glucose with a pyranose ring structure. Hydrolysis with acid gave glucose and acetic acid in equimolar proportions.

A phenylhydrazone was formed with m.p. 136°; $[\alpha]_D^{20} - 13°$ (*c*, 1·3 in H_2O) (Found: $CH_3.CO$, 12·9; N, 8·6. $C_{14}H_{20}O_6N_2$ requires $CH_3.CO$, 13·8; N, 8·97 %.) A small yield of a suspected acetylglucosazone was obtained.

The ester did not form a trityl ether under the conditions used for reaction at a primary alcoholic group (cf. Helferich, 1948). Reaction with periodic acid occurred but no formaldehyde was formed (Reeves, 1941). The evidence, and especially the last two items, indicates that the acetyl residue is associated with $C_{(6)}$ and that the metabolite is 6-*O*-*acetylglucopyranose* (cf. Helferich, 1948; Bell, 1948; Manson & Lampen, 1951). Since it can be obtained in a yield of 20 % of the glucose used it is a major metabolite of this strain. Glucose and sucrose were the only common sugars which yielded an ester. A total of fourteen strains of *B. megaterium* were examined (obtained by courtesy of Dr Ruth Gordon, Rutgers University). Only three produced acetylglucose and the strain used in this work was about four times as effective as the others.

Resting cells of the organism were able to synthesize the ester from glucose, and added pyruvate gave a threefold increase in the amount produced. Arsenite (4×10^{-3} M) gave 40 % inhibition of esterification (of glucose) and pyruvate accumulated.

The results suggest that pyruvate acts here as a source of 'active acetate' in contrast to some other biological acetylations by micro-organisms. The presence of phosphotransacetylase in a cell-free extract suggests the participation of acetylcoenzyme A or acetyl phosphate.

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On the Site of Formation of 5-Hydroxytryptophan. By C. E. Dalgliesh and R. W. Dutton. (*Postgraduate Medical School, Ducane Road, London, W.* 12)

5-Hydroxytryptophan (5-HTP) decarboxylase is a widely distributed enzyme (Gaddum & Giarman, 1956). 5-HTP readily enters cells and penetrates the blood-brain barrier (Udenfriend, Bogdanski & Weissbach, 1956). Such considerations suggest that 5-hydroxytryptamine (5-HT) might be formed in tissues from circulating 5-HTP originating at a single site, such as the liver.

Rat and mouse liver homogenates and slices, and rat and beef liver mitochondrial preparations, incubated with ^{14}C-labelled or unlabelled tryptophan under a wide variety of conditions, have shown no evidence for the conversion of tryptophan to 5-hydroxytryptophan; hydroxylation of kynurenine to 3-hydroxykynurenine (cf. de Castro, Price & Brown, 1956) in mitochondrial preparations occurred readily. Unlabelled 5-HT in the presence of an amine oxidase inhibitor, or carrier 5-hydroxyindoleacetic acid (5-HIAA) were added in the isotopic experiments. 5-Hydroxyindoles were conveniently measured fluorimetrically (Udenfriend, Bogdanski & Weissbach, 1955) using the fluorimeter of Laurence (1957). The 5-HTP decarboxylase activity found for many of the preparations examined was much higher than that found by Gaddum & Giarman (1956), making it unlikely that decarboxylation could be the rate-controlling step in 5-HT formation.

Rat liver perfusion experiments with unlabelled tryptophan, or with ^{14}C-labelled tryptophan and carrier 5-HT and/or 5-HIAA added before and/or after perfusion, gave no indication of any appreciable 5-HTP formation from tryptophan, but both 5-HT and 5-HIAA were converted to derivatives, presumably conjugates, resembling substances noted in carcinoid urines (Dalgliesh, 1956).

Strong indirect evidence that 5-HTP is formed in the argentaffin cells has been obtained from a case of carcinoidosis (Smith *et al.* 1956) in which the presence of metastases in the kidney results in the excretion of much 5-HTP as well as of 5-HIAA and 5-HT. Though the lability of 5-hydroxyindoles and the experimental sensitivity of hydroxylation reactions might account for the negative results with liver preparations, we consider it likely that the argentaffin cells are the main site of 5-HTP formation.

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Intermediates in the Biosynthesis of Aldosterone by Capsule Strippings of Ox Adrenal Gland.

By P. J. Ayres, O. Hechter, N. Saba, Sylvia A. Simpson and J. F. Tait. (*Middlesex Hospital Medical School, London, W.1, and Worcester Foundation for Experimental Biology, Shrewsbury, U.S.A.*)

Wettstein, Kahnt & Neher (1955*a*, *b*) reported that deoxycorticosterone could be converted to aldosterone by adrenal homogenates, but progesterone or corticosterone could not. Rosemberg, Rosenfeld, Ungar & Dorfman (1956), by perfusing isolated adrenals, obtained conversion of progesterone but not of corticosterone or deoxycorticosterone. We have reinvestigated this problem by incubating much smaller quantities of ^{14}C-labelled substrates with capsule strippings of ox adrenals, previously shown to be mainly zona glomerulosa tissue (Ayres, Gould, Simpson & Tait, 1956).

Capsule strippings were shaken under O_2 at 37° in 5 g. batches in 50 ml. Krebs–Ringer phosphate medium containing 13 mM glucose and 3 mM fumarate. The steroid substrates were distributed over the surface of the flask before the medium was added. After incubation for 2 hr., the medium was extracted with chloroform and the extract purified by silica gel chromatography. Cortisol, corticosterone and aldosterone were each subjected to partition chromatography in 60 cm. long kieselguhr columns in two different solvent systems followed by similar purification of their acetyl derivatives on another column. The amounts and specific activities of the acetates were determined in all the fractions of the final column by methods already described (Ayres, Simpson & Tait, 1956). The quoted specific activity is the mean of the individual measurements on those fractions of the final column containing appreciable amounts of the particular steroid. These were constant within the limits of experimental error.

The following substrates were incubated with 10 g. tissue:

(*a*) 50 μg. [4-^{14}C]progesterone, total radioactivity 48 150 cts./min.;

(*b*) 62 μg. [21-^{14}C]deoxycorticosterone, 16 200 cts./min.;

(*c*) 50 μg. [4-^{14}C]corticosterone, 41 250 cts./min.; and also (*d*) double quantities of materials compared with (*c*).

Values ($\pm$10%) for cortisol, corticosterone and aldosterone respectively after incubation were:

(*a*) amounts (corrected for recovery): 21, 113, 61 μg.; specific activities: 6·9, 75, 51 cts./min./μg.;

(*b*) 8·2, 40, 22; $<$3, 43, 43;

(*c*) 4·9, 35, 21; $<$2, 151, 156;

(*d*) 28, 126, 55; $<$1, 103, 88.

The aldosterone diacetate from (*d*) was hydrolysed and the specific activity of the free compound determined by a variety of methods. These measurements confirmed the specific activity of the diacetate.

It has therefore been demonstrated that a major but not necessarily the only pathway of aldosterone biosynthesis lies through progesterone, deoxycorticosterone and corticosterone.

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Differentiation of Cellobiase and 'Carboxymethylcellulase' by Glucono-1:4-lactone. By G. N. FESTENSTEIN. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Carboxymethylcellulose has been frequently employed as a model substrate in studies on cellulase. Relatively stable preparations of an enzyme that hydrolyses carboxymethylcellulose rapidly, but is without appreciable action on filter paper or cotton fibres, can be readily prepared from rumen micro-organisms (G. Halliwell, private communication). It is suggested that the enzyme acting on degraded celluloses or cellulose derivatives, such as carboxymethylcellulose, might be conveniently termed carboxymethylcellulase.

The enzyme preparation was extracted from sheep rumen micro-organisms, using butanol, by the method of Morton (1955), first used for rumen micro-organisms by Halliwell. Carboxymethylcellulose and cellobiose were used as substrates. Reducing sugars were determined by a modified Willstätter hypoiodite method and the colorimetric Nelson copper method (1944) as modified by Somogyi (1952).

Glucono-1:4-lactone has been established as a powerful inhibitor of rumen *o*-nitrophenyl β-glucosidase (Conchie, 1954). In the present study inhibition of carboxymethylcellulase was observed at low concentrations of gluconolactone and increased rapidly with concentration of lactone, but did not exceed 60% inhibition, as determined by reducing sugar estimation. Complete inhibition of cellobiase could be demonstrated.

Paper chromatography of the products of enzymic hydrolysis of carboxymethylcellulose in presence and absence of gluconolactone established that glucose production was very largely, but not entirely, inhibited in presence of gluconolactone at a concentration of lactone that would inhibit cellobiase completely.

Experiments using protozoal invertase and determination of reducing sugar by the modified ferricyanide method of Levvy (1948) showed no inhibition of sucrose hydrolysis by glucono-1:4-lactone.

This problem was suggested by Dr G. A. Levvy. Thanks are due to Dr A. E. Oxford for the protozoal preparation used.

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The Identification of the Sugars of Anthocyanins. By J. B. HARBORNE and H. S. A. SHERRATT. (*John Innes Horticultural Institution, Bayfordbury, Hertford, Herts*)

Earlier studies of the biochemical effects of single gene substitutions upon anthocyanins in plants (Lawrence, 1950) have neglected a detailed consideration of the glycosidic nature of the pigments. The well-known distribution tests (Robinson & Robinson, 1932) only provide limited information regarding the number of sugar residues present and their position of attachment (3-, or 3- and 5-). A more precise knowledge of the glycosidic pattern of the anthocyanins present in genetically analysed plants would add considerably to our understanding of the role of glycosidation in anthocyanin biosynthesis and its relationship to other metabolic processes.

Methods which are now available for the rapid identification of the nature and position of the sugars present in other flavonoid glycosides (Nordström & Swain, 1953; Geissman, Harborne & Seikel, 1956) have been modified for the systematic characterization of anthocyanins. After separation and purification by paper chromatography, the anthocyanins were hydrolysed with acid to give anthocyanidins and sugars. The aglycones were extracted into pentan-1-ol and identified (Bate-Smith, 1954). The aqueous residue was washed with a solution of di-*n*-octylmethylamine in chloroform (10%, w/v) to remove mineral acid (Smith & Page, 1948), concentrated and the sugars identified in the usual way. The positions of the sugars in the anthocyanin molecules were determined by chromatographic comparison with well-characterized pigments and, when two or more sugars were present, by the selective removal of sugar residues by controlled acid hydrolysis. When dealing with acylated pigments, a preliminary treatment with alkali was necessary to remove the acyl groups.

Some results obtained by these methods are already available. Pelargonidin-3-rhamnoglucoside has been identified in pink (bb) flowers of *Antirrhinum majus*. Previously cyanidin-3-rhamnoglucoside had been isolated from magenta (B-) flowers (Scott Moncrieff, 1930). Also the minor

pelargonidin derivative in *Primula sinensis* was found to be a 3-galactoside. The only cyanic pigment which had been fully identified in this plant was malvidin-3-galactoside (Bell & Robinson, 1934). Finally, the pelargonidin, paeonidin and cyanidin glycosides of cultivated diploid potatoes all contain the same sugars, glucose and arabinose. They are present either as a 3-arabinoglucoside or in a glycosidic form which has not previously been described. These data provide further evidence for the genetic control of glycosidation in plant pigments.

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The Specificity of Fungal Anthocyanase. By J. B. Harborne and H. S. A. Sherratt. (*Johns Innes Horticultural Institution, Bayfordbury, Hertford, Herts*)

An enzyme which decolorizes solutions of anthocyanin pigments was found in the culture media of several *Aspergillus* species by Huang (1954, 1956*a*, *b*). The decolorization involved the enzymic hydrolysis of the anthocyanin to anthocyanidin and sugar, followed by the spontaneous transformation of the anthocyanidin to a colourless derivative. It was of interest to determine if the action of this enzyme, anthocyanase, was specific according to the pattern of glycosidation of its substrates to see if it provided a useful means of characterizing unknown anthocyanins.

The enzymic hydrolysis of a range of different anthocyanins has been investigated. Many of the anthocyanins used were isolated by paper chromatography from authentic sources; others became available in the course of other work. A crude preparation of anthocyanase, similar to that used by Huang, was concentrated by precipitation with ammonium sulphate. Solutions of the anthocyanins in lactate buffer, pH 3·9, were incubated with the enzyme at 30°. The rate of hydrolysis was followed visually and by removing samples at intervals and examining them by paper chromatography. Anthocyanidin 3-glucosides, 3-diglucosides, 3-galactosides and 3:5-diglucosides were rapidly hydrolysed and decolorized within an hour. Anthocyanidin 3-arabinoglucosides, 3-rhamnoglucosides and acylated 3:5-diglycosides were much more slowly attacked, and 7–24 hr. elapsed before appreciable loss in colour was observed.

Anthocyanidin monoglucosides were detected as intermediates in the hydrolysis of 3:5-diglucosides and 3-rhamnoglucosides. The enzyme preparation also hydrolysed quercetin-3-rhamnoside and 3-rhamnoglucoside within an hour under the same conditions; with esculin (6-glucosidoxy-7-hydroxycoumarin) hydrolysis was complete within a few minutes. During the hydrolysis of the 3-rhamnoglucosides, the only sugars detected were glucose and rhamnose. The preparation was also shown to possess α-glucosidase activity, when incubated with maltose and α-methylglucose.

The fungal preparation clearly represents a complex mixture of enzymes. However, its activity towards anthocyanins cannot depend solely on the presence of glycosidases of known specificity since emulsin (β-glucosidase) is without action on cyanidin-3-β-glucoside (Huang, 1956*a*).

The crude anthocyanase preparation was kindly provided by the Rohm and Haas Co., Philadelphia, Pa., U.S.A.

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Sulphate Conjugation with Ranol and other Steroid Alchols in Liver Homogenates from *Rana temporaria*. By R. J. Bridgwater and D. A. Ryan. (*Department of Biochemistry and Chemistry, Guy's Hospital Medical School, London, S.E.* 1)

The polyhydric alcohol ranol (Haslewood, 1952) has been conjugated with sodium [^{35}S]sulphate in frog (*Rana temporaria*) liver homogenates. The procedure used was similar to that used for the conjugation of cholic acid with taurine in rat liver homogenates (e.g. Bergström & Gloor, 1954; Bremer, 1955) which we have repeated.

Ranol (0·45 mg. prepared from crystalline ranol sulphate) in ethanol (0·1 ml.) and sodium [^{35}S]-sulphate (1 mg., approx. $1–2 \times 10^6$ cts./min.) were

added to a phosphate buffer (1 ml., pH 7·4) containing $MgCl_2$ (0·00065 M), ATP (1 mg.) and sodium ethylenediaminetetra-acetate (0·5 mg.). Incubation (2 hr./18–22°) of the above solution with a freshly prepared frog liver homogenate, followed by extraction of the mixture with *n*-butanol, resulted in 4–5 % of the radioactivity being found in the butanol layer. Paper chromatography of the butanol extract gave a radioactive spot corresponding with the position of natural ranol sulphate, run as a standard at the same time. Controls (boiled homogenate or omitting ranol) gave not more than 0·2 % of the activity in the butanol and no radioactive spots on paper chromatography.

The apparent ability of frog liver homogenate to conjugate certain other steroid alcohols (3α:7α:12α:24-tetrahydroxycholane, scymnol, cholan-24-ol) with sulphate has also been demonstrated.

Homogenates prepared from the liver of *R. esculenta* did not appear to possess the same degree of conjugating ability for sulphate as those prepared from the liver of *R. temporaria*. It is with a view to measuring differences between species that these enzyme experiments are being carried out.

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The Replacement of Guanine by 8-Azaguanine during Nucleic Acid Biosynthesis in *Bacillus cereus*. By H. G. MANDEL* and R. MARKHAM. (*Agricultural Research Council, Virus Research Unit, Molteno Institute, Cambridge*)

The inhibition of growth of *Bacillus cereus* by 8-azaguanine has been shown to be accompanied by the incorporation of a relatively large amount of this analogue into the ribonucleic acids of this organism (Matthews & Smith, 1956; Mandel, 1956). Since in the case of tobacco mosaic virus grown in the presence of this inhibitor the trace of 8-azaguanine that was incorporated appeared to be present at the expense of guanine (Matthews, 1953), the synthesis of nucleic acids in *B. cereus* during 8-azaguanine inhibition was examined to ascertain whether a similar replacement could be found.

Bacteria grown in a buffered medium of low phosphate content were subdivided into two flasks. One received 20 mg. of 8-azaguanine per l. of medium, and $^{32}PO_4^{3-}$ was then added to both cultures. After incubation of both batches of bacteria for an approximately equal extent of growth, the micro-organisms were harvested, washed with phosphate buffer, extracted with trichloroacetic acid, ethanol and ether, and the nucleic acid-containing residue was treated overnight with N-KOH. The resulting hydrolysate was separated from excess alkali and K^+ by the addition of perchloric acid to pH 5 at 0°, and the nucleotides in the supernatant fluid were separated by paper electrophoresis in formate buffer of pH 3·1 for 7 hr. (20 v/cm.). Cytidylic acid, adenylic acid, guanylic acids-2′ and 3′, 8-azaguanylic acids-2′ and 3′, and uridylic acid, which separated in that order of increasing mobility, were eluted, measured spectrophotometrically, and molar radioactivities were calculated.

The guanylic acid content of bacterial nucleic acid hydrolysates was shown to be decreased by the presence of the analogue. The molar radioactivities of the phosphate in cytidylic, adenylic and uridylic acids were identical but differed sharply from that of the guanylic acid. The analogue seemed to inhibit selectively the biosynthesis of guanylic acid, by approximately the amount of 8-azaguanylic acid synthesized. Under appropriate conditions more 8-azaguanylic acid was synthesized than guanylic acid during the period of inhibition.

*Commonwealth Fund Fellow, on leave from The George Washington University, Washington, D.C., U.S.A.

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The Effect of Soluble Silica on the Respiration of Rat Liver Homogenates. By E. V. ROWSELL and RUTH A. LEONARD. (*Department of Biochemistry, Sheffield University, Sheffield*, 10)

Many workers have considered the possibility that dissolved silica is the pathogenic agent in silicosis (e.g. King, 1950). Accepting this working hypothesis, Kind, King, Pash, Roman & Schmidt (1954) studied the effect of 0·025M soluble silica on the activity of a number of enzymes. They observed only slight inhibitions with lipase, catalase, pepsin, trypsin, erepsin, amylase, choline esterase, and phosphatases. The effect of 0·02M soluble silica, (largely polymerized and colloidal), on the respiration of rat liver homogenates, with a variety of substrates, is now reported.

With aged silica sols inhibition (30–70 %) has been observed with succinate, fumarate, L-malate, β-hydroxybutyrate or DL-lactate as substrate.

With fresh sols, however, very different effects were observed. There was slight inhibition of oxygen consumption with succinate, β-hydroxybutyrate and DL-lactate. With fumarate and L-malate there was little difference in the initial respiration rate but, whilst this diminished in the controls, with silica the rate was maintained, and the total oxygen consumption was thereby much greater (more than twofold in some experiments). This effect was not observed with the other substrates tested. (DPN was added with fumarate, malate, β-hydroxybutyrate and lactate.)

When fumarate or L-malate are oxidized enzymically oxalacetate accumulates and inhibits the reaction. It is assumed that this occurred in the control experiments. For the assay of malic dehydrogenase in tissue extracts some method must be used to remove the oxalacetate (e.g. transamination with L-glutamate) so that the oxidation can proceed (Potter, 1946). A possible explanation of the continued oxygen consumption in the presence of soluble silica is that silica in some way facilitates the removal of oxalacetate.

Oxygen consumption with oxalacetate as substrate was slightly inhibited by 0·02M silica (fresh sols.). Other possibilities for the removal of oxalacetate in the presence of silica (e.g. accelerated decarboxylation or adsorption on to the polymerized silica) have not yet been examined.

In all experiments the tissue homogenate was precipitated within 2–3 min. of addition to medium containing 0·02M silica. It is remarkable that under such conditions undiminished or enhanced rates of respiration could be observed.

0·002M silica (molybdate reactive, non-polymerized) had no effect on the respiration with succinate, fumarate, or β-hydroxybutyrate.

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DEMONSTRATIONS

The Aminco-Bowman Spectrophotofluorometer. By R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

Existing fluorimeters utilize only a few wavelengths of a mercury lamp for activating fluorescence in organic compounds. The present new instrument is a commercial adaptation (The American Instrument Company, Silver Spring, Maryland) of a prototype developed by Dr R. L. Bowman (Bowman, Caulfield & Udenfriend, 1955) of the National Institutes of Health, Bethesda, Md., from ideas originally conceived by Dr B. B. Brodie and Dr S. Udenfriend of the National Heart Institute. This instrument both activates and measures fluorescence throughout the visible and ultraviolet regions, and allows the identification and determination of many organic compounds, which fluoresce, in quantities of 0·01–1·0 μg./ml.

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A Stable Single Beam Fluorimeter covering Excitation Wavelengths less than 365 mμ.

By D. J. R. Laurence*. (*Postgraduate Medical School, Ducane Road, London, W.* 12)

The Osram (Germany) Spectral lamp type HgS differs from many equivalent types in showing inherent stability, i.e. the arc current is constant when a constant a.c. voltage is applied across the lamp and series ballast. For correct matching with a standard constant-voltage transformer a resistive, rather than inductive, ballast is needed, and many lamp types become very unstable when operated in this way from a constant-voltage transformer with series resistance. The HgS lamp in the same circuit shows no inherent instability and only 0·5% change in light intensity for a 20v difference in the voltage applied to the constant-voltage transformer. The stability is therefore sufficient for use in a single beam fluorimeter and a simple apparatus of this nature is demonstrated.

As the lamp has an inner bulb of special glass and an outer bulb with a hole as a window, appreciable emission in the wavelength region 250–360 mμ. is available. The light source is focused on to a quartz test tube by means of a pair of plano convex lenses, the light between the lenses being parallel to allow the use of interference filters for the exciting light. A similar pair of lenses and a filter are used in the fluorescence beam, which is at right angles to the excitation beam. The fluorescence intensity is measured with an E.M.I. photomultiplier and the sensitivity of the apparatus is increased by screens placed as required to shield stray light and reduce the blank value. The apparatus permits excitation of fluorescence of, for example, tryptophan metabolites; thus 0·02 μg./ml. of 5-hydroxytryptamine may be estimated in the apparatus (cf. Dalgliesh & Dutton, 1957).

A commercial fluorimeter based on the HgS lamp is now available from the Locarte Company, London, S.W. 7.

* Now at the Chester Beatty Research Institute, Fulham Road, London, S.W. 3.

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A Combined Spectrophotofluorimeter and Double Monochromator Spectrophotometer.

By R. J. Bartholomew, C. E. Dalgliesh and I. D. P. Wootton. (*Postgraduate Medical School, Ducane Road, London, W.* 12)

Workers at the National Institutes of Health at Bethesda have demonstrated the great potential power of spectrophotofluorimetry as a tool for biochemical research (e.g. Bowman, Caulfield & Udenfriend, 1955; Udenfriend, Bogdanski & Weissbach, 1955). A suitable instrument must allow determination of an 'excitation spectrum' by measuring fluorescence at any selected wavelength as a function of the wavelength of the exciting light, and of a 'fluorescence spectrum' at any selected exciting wavelength by measuring fluorescence as a function of the wavelength of the fluorescent light. By selecting wavelengths for excitation and for measurement of fluorescence appropriate to the compound under investigation, quantitative determinations of considerable specificity can be made of numerous biologically important substances.

The spectrophotofluorimeter demonstrated is designed to allow irradiation of a liquid with monochromatic light of any desired wavelength in the range 220–1000 mμ., and measurement of resulting fluorescent light at any wavelength within the same range. Two quartz prism monochromators are rigidly mounted together on a common base, parallel, and with all slits at the same end of the assembly. The slit control and scale, and the wavelength control and scale of the primary (rear) monochromator are mounted on the same panel with, and immediately above, the corresponding controls of the secondary (front) monochromator. Light from a 200w xenon arc is focused on the entrance slit of the primary monochromator, and the emergent monochromatic beam is deflected and focused on to the material under examination, contained in a quartz cell placed at the entrance slit of the secondary monochromator. The fluorescent light passes through the secondary monochromator and the emergent beam passes to a photomultiplier measuring system.

On replacing the fluorimeter cell by a prism, the instrument is converted into a double monochromator spectrophotometer, for use of which a standard cell compartment and cell carrier are incorporated. A hydrogen arc and tungsten filament are also available as alternatives to the xenon arc.

Development of this instrument was made possible by the generous support of Mr W. H. Storey and Messrs Unicam Ltd. of Cambridge, to whom we are greatly indebted. We thank the staff of the Biophysics Unit of the Postgraduate Medical School for their able assistance.

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[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **358th Meeting** *of the Biochemical Society was held in the Department of Biochemistry of the London Hospital Medical College on Saturday,* 19 *January* 1957, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Properties of Partially Purified L-Galactono-γ-lactone Dehydrogenase. By L. W. MAPSON and E. BRESLOW. (*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research*)

The terminal step in the biosynthesis of L-ascorbic acid in peas has been shown to be the enzymic oxidation of L-galactono-γ-lactone (Mapson, Isherwood & Chen, 1953). An enzyme catalysing the oxidation of this lactone to ascorbic acid in cauliflower has been solubilized and purified tenfold from an acetone powder extract of cauliflower mitochondria. Of many hydrogen acceptors tested, the solubilized enzyme is able to reduce only phenazine methosulphate and cytochrome *c*. Fractionation has therefore been followed by a spectrophotometric method based on the rate of reduction of indophenol blue by enzymically-reduced phenazine under anaerobic conditions, while both cytochrome *c* and phenazine have been used as acceptors in studies of other properties.

p-Chloromercuribenzoate inhibition and glutathione reactivation indicate that the enzyme requires sulfhydryl groups for its activity. Neither DPN nor TPN stimulate the reduction of phenazine and neither are reduced by the enzyme in the presence of substrate. The phenazine and cytochrome systems are insensitive to cyanide, EDTA, 8-hydroxyquinoline and pyrophosphate, but the reduction is inhibited after incubation with 10^{-3} M *ortho*phenanthroline in the absence of substrate.

Preliminary experiments indicate that the enzyme may be a flavoprotein. The reaction with ferricytochrome *c* as electron acceptor was inhibited by both atebrin (60% at 10^{-3} M) and riboflavin (56% at 10^{-4} M). The reduction of phenazine was not inhibited by atebrin or riboflavin, although it was inhibited by acriflavine.

Differences between the phenazine and cytochrome reductions are tentatively explained by assuming different electron pathways for the two systems. Phenazine reduction is markedly stimulated by increasing phosphate concentration up to 0·08 M, although it has not yet been possible to demonstrate an absolute phosphate requirement for this system. The cytochrome system, however, is strongly inhibited by phosphate concentrations higher than 0·01 M. The effect of phosphate on cytochrome *c* reduction by L-galactono-γ-lactone dehydrogenase appears to be due to competition between the phosphate and the cytochrome *c* for the dehydrogenase, a relation similar to that existing between pyrophosphate and the adenosine phosphates and cytochrome *c* reduction with other metalloflavoproteins (Mahler, 1953).

All attempts to resolve the enzyme into apoenzyme and prosthetic group by acid treatment and to re-activate by either FMN or FAD have so far been unsuccessful.

The enzyme appears to be specific for L-galactono-γ-lactone since this was the only aldonic acid lactone tested which was oxidized at an appreciable rate. L-Gulono-, D-mannono-, D-glucono-, D-galactono- and D-altrono-γ-lactones were either not oxidized or oxidized only very slowly. L-Galactonic acid and D-glucurono-γ-lactone were not oxidized.

The work described in this paper was carried out as part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research.

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Some Aspects of the Effect of the Initial pH on the Behaviour of Glucose in the Amino Acid–Glucose Interactions. By S. LEWIN. (*Physical Chemistry Laboratory, South-West Essex Technical College, London, E.* 17)

Investigation of the interaction of glucose with several amino acids (histidine, glutamic acid, α-alanine, glycine, α-aminobutyric acid, lysine, ornithine, valine, leucine and norvaline) in the two initial pH ranges of 6·5 to 9 and the pH range over *ca.* 10·3 shows a clear-cut difference in that a series of steady pH states is always obtained in the first, lower, pH range provided the amino acid concentration is above 0·015M (Lewin, 1955, 1956*a*, *b*; Lewin & Kosinski, 1956; Lewin & Fox, 1956), whereas at the higher pH range only continuous pH depressions are recorded.

Chromatographic analysis gives a clear contrast in the behaviour of glucose solutions initially adjusted to pH values in the two respective pH ranges in that only at the higher pH range is transformation of glucose found. Thus, glucose solutions initially adjusted to pH 8·4 with NaOH and kept for over a month at 40° give only glucose spots. Similarly, glucose–amino acid solutions with an initial pH range of up to *ca.* pH 9 give spots for glucose, but not for other sugars, during the first four steady pH states (see also, Gottschalk & Partridge, 1950). Also, glucose solutions, buffered with phosphate, in the lower pH range, e.g. pH 7·80 at 40°, retained their initial pH value for over 30 hr. and gave glucose spots only, even after 3 days. But glucose solutions adjusted to the higher pH range, e.g. M glucose adjusted with NaOH to pH 10·2 at 40° gave, after several days, spots for both glucose and fructose. The change is compatible with a mechanism proceeding via glucose anion; the absence of such changes in the lower pH range is attributable to the minute concentration of the glucose anion in that region, e.g. $<10^{-4}$ at pH 8·1.

The absence of spots for other sugars, in the glucose–amino acid interactions in the lower pH range, can be taken as evidence that this reversible interaction is associated with glucose itself and not with a degradation product of it. The continuous pH drop found in the higher pH range is consistent with the comparatively high buffering capacity pH drop which accompanies the transformation in the high pH value glucose solutions.

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Some Aspects of the Interaction of Glycylglycine with Glucose. By S. LEWIN and J. S. MCCALL. (*Physical Chemistry Laboratory, South-West Essex Technical College, London, E.* 17)

The interaction of glycylglycine with glucose was examined by several authors, in each case at a single temperature, including Katchalsky (1941) who interpreted his results in terms of a reversible interaction of one molecule of glucose with one molecule of the anion of glycylglycine and who was the first to assign to the reaction a pH independent equilibrium constant.

Our investigation of this reaction—over the pH range of 4 to 9, using glucose concentrations up to 1·3M, at 20°, 30° and 40°—by following the pH depressions and using Lewin's (1956) formulation shows the coexistence of reversible interactions of one and two glucose molecules with one molecule of the anion of glycylglycine, as well as a very small interaction between glucose and the zwitterion, in the first steady pH state obtained. The overall reaction was found to be parallel to other amino acid–glucose interactions (cf. previous abstract) in being also exothermic and in showing continuous pH depressions in the initial pH range of over *ca.* pH 10·3.

This reaction can, however, be distinguished from other amino acid–glucose interactions in that (*a*) the first steady pH state obtained lasts much longer than the first steady state in the amino acid–glucose interactions. Indeed, its duration covers the period during which the first three steady pH states are observed in the amino acid–glucose interactions, and (*b*) the practical absence of any development of brown colour even after a month at 40°.

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Toxic Liver Injury. Inhibition of Uptake of Labelled Amino Acids into Liver Protein *in vivo* by Dimethylnitrosamine. By P. N. MAGEE. (*Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey*)

Dimethylnitrosamine causes acute centrilobular necrosis of the liver in rats with minimal damage to other tissues (Barnes & Magee, 1954) and produces malignant hepatic tumours after prolonged administration (Magee & Barnes, 1956). It is rapidly metabolized *in vivo* (Dutton & Heath, 1956), probably only by the liver and it is distributed uniformly throughout the body water, with no preferential accumulation in the liver (Magee, 1956).

An early histological change in the liver after a necrotizing dose of dimethylnitrosamine is reduction in cytoplasmic basophilia in the central and midzones. In view of the current theories on the relation between nucleic acids and protein synthesis, it was thought that some alteration in protein turnover might be induced in the liver.

Mixed ^{14}C-labelled amino acids were injected intravenously into rats treated with dimethylnitrosamine and the specific radioactivities of the proteins from liver and some other tissues were compared with those of untreated control animals, using the methods of T. S. Work and J. L. Simkin (personal communication). In rats 6 hr. after treatment, the specific activity of the liver protein was reduced to rather less than 50% of that of the controls, while the activities of the kidney, spleen and pancreas proteins were not significantly altered. This reduction in the liver activity might have been due to a disorder in the circulation (cf. Henriques, Henriques & Neuberger, 1955). The specific activities of the free amino acids of the liver and the liver protein were therefore measured 3 hr. after dimethylnitrosamine, and the protein activity was found to be reduced by about 50% while the free amino acid activity was not significantly changed.

It seems therefore that there is some failure in the mechanism of incorporation of the amino acids into liver protein rather than a failure of their supply to the hepatic cells, and this is supported by the work of Stoner (1956) who found no reduction in liver blood flow in the rat during the first 24 hr. after the same dose of dimethylnitrosamine. Histological evidence of damage to the liver is minimal or absent 3 hr. after administration. The reduction in uptake of amino acids may be an early non-specific manifestation of cell death but it may be related to the fundamental biochemical lesion.

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The Fatty Acid Composition of the Sterol Esters and Triglycerides in the Plasma of Lactating Cows. By A. K. LOUGH, G. A. GARTON and W. R. H. DUNCAN. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Lipids prepared by extraction with ethanol-ether from a pooled sample of the plasma of six lactating pasture-fed cows were chromatographed on a silicic acid column (Fillerup & Mead, 1953; Mead & Fillerup, 1954) to yield sterol esters (81·8%), triglycerides (7·5%), free sterols (6·4%), phospholipids (3·7%) and unidentified lipids (0·6%). The fatty acids of the sterol esters and the triglycerides were submitted to reversed-phase partition chromatography (Howard & Martin, 1950) before and after hydrogenation of the unsaturated components (cf. Popják & Tietz, 1954). From the chromatographic results the composition of each group of acids was calculated. Spectrophotometric analysis following alkali-isomerization was employed to determine the linoleic acid content of the glyceride fatty acids and to confirm the proportions of polyethenoid components deduced chromatographically to be present in the fatty acids of the sterol esters.

The composition of the fatty acids of the sterol esters was (in mols. %): myristic 4·4, palmitic 1·6, stearic 2·1, arachidic 0·8, palmitoleic 11·2, oleic 6·1, linoleic 35·8, linolenic 32·0, arachidonic 6·0; giving iodine value 192·4 as compared with 189·5 by direct determination. The component fatty acids of the triglycerides were (in mols. %): myristic 4·6, palmitic 29·9, stearic 30·6, myristoleic 1·8, palmitoleic 7·2, oleic 21·5, linoleic 1·5, gadoleic 2·9; giving iodine value 34·3 as compared with 36·3 by direct determination.

Similar findings to those reported above were obtained on the sterol esters and triglycerides in the

plasma, taken at slaughter, from one other lactating cow. In neither analysis was there any chromatographic evidence suggesting the presence of *n*-acids possessing an odd number of carbon atoms such as have been detected in very small amounts in bovine milk fat (see, for example, Shorland, Gerson & Hansen, 1955*a*, *b*; Garton & Lough, 1957), though it is possible that traces were present.

These results differ in several respects from those obtained by Kelsey & Longenecker (1941) who determined, by ester fractionation, the component fatty acids of the cholesterol esters and triglycerides in the plasma of pasture-fed cows; in their investigation, however, the complete separation of the two classes of lipid (by selective enzymic hydrolysis of the triglycerides) is open to doubt. In addition, Kelsey & Longenecker reported the presence of 10·7% of free fatty acids (mainly palmitic and oleic) in their plasma lipid preparation, whereas in the present study, only traces of free fatty acids were found in the plasma lipids (cf. Channon & Collinson, 1929).

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Polyphenoloxidase Oxidation of Catechin, and an Initial Comparison of the Polymer obtained with two Tannins. By D. E. Hathway and J. W. T. Seakins. (*British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey*)

The formation of polymers and hydrogen peroxide during the autoxidation at 35° and pH 6–8 of catechin and of related 3′:4′-dihydroxyflavans has been studied by measurement of oxygen absorption, and by the elementary analyses, absorption spectra and reactions of the dialysed polymers (Hathway & Seakins, 1957). The evidence obtained, together with that provided by spectroscopic study of intermediates produced by silver oxide oxidation (Hathway & Seakins, 1955), support the quinone polymerization mechanism for catechin autoxidation.

Mushroom, potato and tobacco phenoloxidases have now been found to oxidize catechin at a faster rate and lower temperature to a product precisely similar to the autoxidation polymer; the rate of oxygen absorption being proportional to enzyme concentration. Since catechin autoxidation proceeds by quinone polymerization, the similar enzymic oxidation therefore involves the polyphenoloxidase activity (Keilin & Mann, 1938) of the enzymes.

Since *Acacia catechu* (Leguminosae) heartwood and *Uncaria gambir* (Rubiaceae) leaves afford extracts (40% tannin, hide-powder test) which contain large quantities of *epi*catechin and catechin respectively, the residual aqueous solutions were examined after exhaustive solvent extraction, and found to contain 20% tannin. Two-dimensional paper chromatograms in both cases revealed a single phenolic component at the origin. Deionization of the residual solutions precipitated the tannins, which were separated in the centrifuge. The absorption spectra of these tannins at pH 8 were similar to those of catechin autoxidation and polyphenoloxidase oxidation polymers, exhibiting maxima at 270, 410 and 500 mμ.

On account of polyphenoloxidase oxidation leading to catechin polymer and the occurrence of similar tannins amongst the extractives of two tanning materials, it is now suggested that these tannins may be formed from catechin epimers by polyphenoloxidase oxidation mainly during harvesting and storage, and the heterotypic behaviour of this enzyme system is recalled. Considerable autoxidation of catechin may also occur during extraction.

A flavonoid fraction from *Acacia catechu* was found to contain in addition to quercetin (Perkin, 1897) two flavonols, chromatographically indistinguishable from fisetin and quercetagetin. Authentic quercetagetin was isolated from *Tagetes erecta* flowers.

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A New Amino Sugar in Acid Hydrolysates of Chondroitin Sulphate from Hyaline Cartilage.

By HELEN MUIR.* (*Medical Unit, St Mary's Hospital, London, W. 2*)†

Examination by two-dimensional chromatography of the amino acids in hydrolysates of a chondroitin sulphate complex from hog laryngeal cartilage (Muir, 1956) showed the presence of a ninhydrin-positive spot with reducing properties which had the same mobility as galactosamine in the phenol–NH_3 direction of paper chromatograms, but which moved much more slowly in the butanol–acetic acid direction. The polysaccharide was hydrolysed in sealed tubes at 100° and the spot appeared when the HCl concentration was varied between 2 and 6 N and when hydrolysis was prolonged. It was also obtained from hydrolysates of commercial chondroitin sulphate which was almost free of amino acids. By over-running one-dimensional chromatograms with butanol–acetic acid–water (Partridge, 1948) the unknown substance separated very well from the galactosamine in the hydrolysate or from added glucosamine. The $R_{glucose}$ values on Whatman no. 54 paper were glucosamine 0·73; galactosamine from hydrolysates 0·67, unknown 0·27, authentic galactosamine 0·67.

Both the galactosamine and the unknown substance gave positive Elson and Morgan reactions, but did not react directly with Ehrlich's reagent. Qualitatively they reacted in the same way with aniline hydrogen phosphate, benzidine, naphthoresorcinol, diphenylamine and ninhydrin and showed the same electrophoretic mobility on paper from pH 4 to 8·4. It thus seems unlikely that the unknown substance was the disaccharide chondrosine, but was more probably a 2-amino sugar with the same ionizable groups as galactosamine.

Oxidative deamination with ninhydrin (Stoffyn & Jeanloz, 1954) showed that both galactosamine and the unknown gave pentoses. Mixtures of the pentose obtained from the unknown with either ribose or arabinose could be separated on paper chromatograms run in butanol–acetic acid–water. Chromatographic separation of xylose and lyxose is not satisfactory, but they were separated by paper electrophoresis in borate buffer by a modification of the method of Consden & Stanier (1952). The pentose from the unknown sugar was thus separated from xylose when mixed with an authentic specimen, but it could not be separated from an authentic specimen of lyxose. The pentose obtained from galactosamine, which is known to be lyxose, behaved similarly. As the unknown sugar gives the same pentose as galactosamine and has the same reactive groups, it is probably the epimeric amino-sugar talosamine.

* Empire Rheumatism Council Fellow.

† Present address: Postgraduate Medical School, Ducane Road, London, W. 12.

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Cholesterol and Liver Vitamin A in Male and Female Rats.

By B. GREEN, A. A. HORNER, J. S. LOWE and R. A. MORTON. (*Department of Biochemistry, University of Liverpool*)

It has recently been reported from this laboratory (Kantiengar & Morton, 1955), incidentally to other work, that the bulked livers from five adult male rats fed a dietary supplement of cholesterol (2 %) for 8 weeks, contained 35 % less vitamin A than the bulked livers from five control animals.

Further investigations of the relationship between cholesterol feeding and liver vitamin A levels have now been made. Individual livers have been examined, and more animals used. The results have been statistically analysed and their significance decided by the 't' test. It has been established that there is a sex difference in the effect of cholesterol-feeding on liver vitamin A storage in the rat.

In the case of females, neither adults, nor young rats fed the cholesterol supplement from weaning, showed any significant lowering of liver vitamin A stores. Both young and adult males fed cholesterol exhibited lower liver vitamin A levels than their controls, and the differences were statistically significant at the 0·01 level of probability. The effect was greater in sexually immature males than in adult males. The mean depressions of liver vitamin A, as compared to the mean control levels, were 46 % for young males and 16 % for adult males.

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Competitive Inhibitors of Phosphoglucose Isomerase. By C. W. PARR. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

The interconversion of glucose 6-phosphate and fructose 6-phosphate, by phosphoglucose isomerase from various mammalian tissues, is strongly inhibited by preparations of 6-phosphogluconate (Parr, 1956). Further work has shown that isomerases from the potato and from *Escherichia coli* are also inhibited by 6-phosphogluconate, and it has been demonstrated by the method of Lineweaver & Burk (1934), using both a potato and a beef-liver enzyme, that the inhibition is competitive.

Glucose, gluconic acid, sorbitol, glucose 1-phosphate and inorganic phosphate, amongst other substrates tested, had little or no inhibitory effect. However, a preparation of sorbitol 6-phosphate, made from glucose 6-phosphate by reduction with sodium borohydride as described by Abdel-Akher, Hamilton & Smith (1951), was found to act strongly as a competitive inhibitor of both the potato and the liver isomerases. Very recently Wolfe & Nakada (1956) have reported that the phosphoglucose isomerase from *E. coli* is inhibited by glucosamine 6-phosphate.

Topper (1956) has given evidence, from enzymic reactions carried out in the presence of deuterium oxide, which suggests the intermediate formation of the straight chain enediol, and Bruns & Okada (1956), as the result of thermodynamic considerations, have suggested that this enediol form of the hexose phosphate is associated with the enzyme to form the enzyme-substrate complex. The three inhibitors so far discovered are identical at C_2 to C_6 with the proposed enediol intermediate (and also with the two hexose phosphates involved in the reaction), although interesting variations occur in the groupings at C_1 and C_2.

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Some Observations on the Behaviour of Steroid Conjugates on Alumina. By J. J. BARLOW* (introduced by A. E. KELLIE). (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.* 1)

Steroid metabolites are excreted in the urine mainly as glucuronides and sulphates. Although it is possible to discriminate between these two forms of conjugation by specific hydrolytic procedures and to separate them physically by solvent partition after acetylation (Kellie & Wade, 1956) neither process gives the steroid conjugates in unmodified form. The former liberates free steroids and the latter yields acetylated glucuronides which can no longer be hydrolysed by β-glucuronidase or oxidized by bismuthate. In the present communication an alternative method is described which, taking advantage of adsorption on alumina, separates glucuronides and sulphates in unmodified form.

Urinary steroid conjugates (equivalent to one-tenth day) are extracted by the method of Edwards, Kellie & Wade (1953) and the ether–ethanol solution is poured directly on to a column of neutral alumina (20 g.; moisture content 2%) made up in benzene (height of column 20 cm., internal diam. 1 cm.). The conjugates are firmly adsorbed and the ether–ethanol filtrate contains 1% or less of the urinary 'total ketosteroids' (Brooks & Norymberski, 1953). The column is then washed consecutively with ethanol (200 ml.) and 50% (v/v) aqueous ethanol (200 ml.) The material eluted by these solvents contains no glucuronic acid nor are the 17-oxosteroids present liberated by incubation with β-glucuronidase. Hydrolysis by continuous extraction with ether at pH 1 liberates an amount of 17-oxosteroid which corresponds closely to the figure for 17-oxosteroid sulphate obtained by alternative methods.

The steroid conjugates remaining on the column can be quantitatively eluted by sodium phosphate/citrate buffer solution (M/25, pH 6). The conjugates in the eluate can be hydrolysed by β-glucuronidase and the amount of 17-oxosteroid liberated corresponds closely to the amount of 17-oxosteroid glucuronides determined by other methods. The 17-oxogenic steroid glucuronides, which are also recovered quantitatively from the column, are largely confined to this fraction. The glucuronide fraction obtained by this method is only about one-tenth the weight of the original urine conjugate extract.

* U.S.A. Public Health Research Fellow of the National Cancer Institute.

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Fructose Formation in Goat Placenta. By M. W. NEIL, D. G. WALKER and F. L. WARREN. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

Walker (1956) reported the results of experiments in which massive injections of various sugars were made into the maternal and foetal circulations of the pregnant goat, in order to study the transport of these sugars across the placenta. In general he found that the placenta was fairly permeable to aldoses, but that the transmission of ketoses was much restricted. These experiments have now been extended to include sorbitol injection, and perfusion of the placenta with saline containing sorbitol.

In contrast to glucose, injections of sorbitol into the maternal circulation produce only small rises in foetal plasma fructose concentration, whereas distinct rises in maternal plasma fructose and glucose occur. Injection of sorbitol into the foetal circulation produces a rise in foetal plasma fructose but not glucose. Fructose injection into the maternal circulation produces a rise in maternal plasma glucose, but a corresponding injection into the foetus does not raise the foetal plasma glucose.

Thus the mechanism whereby plasma glucose is formed from injected fructose in the maternal goat is not operative in the foetus, representing an interesting difference between adult and foetal metabolism.

Perfusion of the placenta with dextran-saline containing sorbitol did not show an appreciable increase in the rate of fructose formation over that observed in a sorbitol-free control perfusion. This is perhaps surprising since sorbitol dehydrogenase has been found to be present in goat placenta. This enzyme is also present in the placenta of the sheep and cow, but not in that of the pig which is also an ungulate.

Injection of glucose into the maternal circulation has been followed by analyses of cotyledons for phosphoric acid and its esters. Only fructose-1, 6-diphosphate accumulated to a marked extent.

These observations have a bearing on the problem as to how the fructose in the blood of foetal ungulates arises. It is known that the sheep placenta is a site of fructose formation from glucose (Huggett, Warren & Warren, 1951), and the same has been found to be the case in the goat.

The absence of sorbitol dehydrogenase in the placenta of the pig makes it difficult to explain the formation of fructose in ungulate placentae by the mechanism proposed by Hers (1956) for the formation of fructose from glucose via sorbitol in sheep seminal vesicles.

While the fact that only fructose-1, 6-diphosphate accumulates in placental tissue after glucose injection may support the concept that the fructose formation occurs via phosphorylated intermediates (Winterton, 1949), it may also be interpreted simply as a reflexion of the increased carbohydrate metabolism in the tissue.

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Isolation and Purification of a Soluble Cytochrome from a Fluorescent Pseudomonad. By MARGOT KOGUT (introduced by S. R. ELSDEN). (*A.R.C. Unit for Microbiology, Department of Microbiology, University of Sheffield*)

The components of the cytochrome system in different strains of the *Pseudomonas fluorescens* species group have been described by a number of workers (Fujita & Kodama, 1934; Stanier, Gunsalus & Gunsalus, 1953; Lenhoff & Kaplan, 1953; Smith, 1954).

Spectroscopic observation of whole cells of a fluorescent Pseudomonad, strain KB1 (Kogut & Podoski, 1953) showed the following absorption bands: 522–530, 550–558 and 560–566 mμ. Smith (1954) has recorded for the difference spectrum of whole cells of *Ps. fluorescens*, peaks at 424, 523, 552, 560 and 580 mμ., and Stanier *et al.* (1953) for absorption spectra of cell-free extracts, peaks at 422, 520, 552 and 560–562 mμ. Difference spectra of whole cells of KB1 in 30% serum albumen had peaks at 427–430, 527, 555 and 560–562 mμ.

A soluble cytochrome has been extracted from dried cells with 5% (w/v) borax, and showed absorption maxima at 417, 524 and 553 mμ. After concentrating, the pigment was purified by fractionation between 0·35 and 0·65 saturated neutral ammonium sulphate. The preparation was very viscous and had high absorption in the ultraviolet region. Either precipitation at pH 3 with acetic acid, or digestion with deoxyribonuclease lowered the viscosity and reduced the absorption at 260 mμ. Considerable loss of pigment resulted from either treatment. Further purification was achieved by zone electrophoresis in borate-phosphate buffer,

pH 8·5. (Flodin & Porath, 1954; Campbell & Stone, 1956).

The most highly purified material, judged by the ratio of absorption at 280 mμ. to that at the Soret peak, had the following properties: absorption maxima at 365, 410 and 525–540 mμ. when oxidized; 417·5, 524·5 and 553 mμ. when reduced; not appreciably autoxidizable; heat-stable; no combination with CO or KCN; alkaline pyridine and cyanide haemochromogens (Kamen & Vernon, 1955) were similar to those derived from other *c*-type cytochromes; isoelectric point below pH 6·6; oxidation-reduction potential between the ranges of the ferri-ferrocyanide and the ferro-ferri-oxalate systems; preliminary measurement with 2:6-dichlorophenol-indophenol at pH 6·9 indicating E'_0 value of about 235 mv.

The pigment thus appears to be a cytochrome of the *c*-type, but differs in some of its properties from all others so far described. Adopting the nomenclature of Scarisbrick (1947) it is proposed to call it Pseudomonas cytochrome 553.

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Configuration of Amino Acids formed by Reductive Amination in *Bacillus subtilis*. By P. C. Shah and H. K. King. (*Department of Biochemistry, University of Liverpool*)

When alanine is formed from pyruvate and ammonia by *Bacillus subtilis* (Fairhurst, King & Sewell, 1956) the racemic DL mixture is obtained. This raises the question whether the reductive amination enzyme system performs a symmetrical synthesis, or whether the L isomer is initially produced and subsequently racemized by the alanine racemase reported to occur in *B. subtilis* and other bacteria (Wood & Gunsalus, 1951). We have prepared α-keto-*iso*valeric acid and α-keto-*iso*caproic acid (the keto analogues of valine and leucine respectively) and incubated their ammonium salts with washed suspensions of *B. subtilis*. Valine and leucine were formed biosynthetically, and in each case the amino acid was separated from the medium by means of a cation-exchange resin prior to purification by recrystallization from alcohol–water mixtures. The $[\alpha]_D$ values in both cases were close to those of authentic samples of the L isomers, and manometric measurements with a D-amino acid oxidase showed that not more than 1 % of the D isomer could be present. The suspensions of *B. subtilis* were further shown to possess a racemase active with alanine but not with valine or leucine. The activity of this enzyme was sufficient to racemize alanine as rapidly as the reductive amination system could form it. The DL-alanine produced by reductive amination of pyruvate thus probably arises by racemization of an optically active primary product.

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Inhibition of B_6-Enzymes by Toxopyrimidine Phosphate (4-Amino-5-hydroxymethyl-2-methylpyrimidine *O*-Phosphate). By Barbara G. Haughton and H. K. King. (*Department of Biochemistry, University of Liverpool*)

Structural analogues of pyridoxal phosphate are known, but none described so far are of much practical use as competitive inhibitors in the investigation of B_6-enzymes. The report of Makino & Koike (1954) that toxopyrimidine phosphate strongly inhibited the tyrosine decarboxylase of *Streptococcus faecalis R* by competition with pyridoxal phosphate was therefore of interest. We have examined the potentialities of this compound by investigating its action on several bacterial enzymes known to require pyridoxal phosphate.

Toxopyrimidine phosphate did not inhibit glutamic acid or arginine decarboxylases, tryptophanase, or glutamic acid–alanine transaminase, in

cell-free extracts of *Escherichia coli*. No attempt was made to resolve these enzymes but toxopyrimidine phosphate was used at a concentration of 5×10^{-4}M, 200 times greater than that reported to inhibit tyrosine holodecarboxylase, and 100 times greater than the pyridoxal phosphate content of the enzyme preparations used.

Crude tyrosine decarboxylase (acetone powder of *Streptococcus faecalis*) was inhibited to the extent of 40%. The enzyme was extracted and considerable separation of apoenzyme and coenzyme achieved by precipitation with ammoniacal ammonium sulphate (Epps, 1944). When sufficient coenzyme was added for half-maximal activity, toxopyrimidine phosphate inhibited decarboxylation by only 54%, when added at a concentration 30 000 times in excess of the coenzyme. This inhibition rose to 92% when the inhibitor was added before the coenzyme. Simultaneous addition of coenzyme and inhibitor gave intermediate values. The affinity of the enzyme for toxopyrimidine phosphate was much lower than for pyridoxal phosphate although inhibition was not strictly competitive.

The leucine decarboxylase of *Proteus* (Ekladius & King, 1956) might be a specially favourable case for demonstrating the action of toxopyrimidine phosphate, since the coenzyme normally dissociates spontaneously from the apoenzyme on addition of the substrate. Little inhibition by toxopyrimidine phosphate was, however, observed.

Toxopyrimidine phosphate is therefore little more effective than other inhibitors of B_6-dependent systems. This may be due to the fact that in these enzymes combination between coenzyme and apoenzyme is not readily reversible.

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Serum Proteins and Protein-Bound Carbohydrate in Relation to Tissue Breakdown and Regeneration. By J. W. KEYSER, W. J. JENKINS, G. H. T. LLOYD, C. PALLIS and P. G. SANDERS.
(*Departments of Pathology and Neurology, Welsh National School of Medicine, Cardiff*)

To investigate the significance of elevated serum concentrations of protein-bound carbohydrate (PBC) and α-globulins (cf. Seibert, Seibert, Atno & Campbell, 1947; Shetlar, Bryan, Foster, Shetlar & Everett, 1949; Shetlar, 1952; Shedlovsky & Scudder, 1942), we studied (*a*) patients with myopathies, motor neurone disease and allied conditions; (*b*) patients with injuries (fractures, etc.); (*c*) normal women throughout the menstrual cycle. Total protein was separated by alcoholic precipitation (Keyser, 1952) and mucoprotein according to Winzler, Devor, Mehl & Smyth (1948), PBC being estimated with orcinol (Sørensen & Haugaard, 1933; Rimington, 1940).

In series (*a*) most patients classified clinically as actively wasting had elevated PBC levels, unlike most of those classified as probably inactive. The difference between the means for the two groups is apparently significant in respect of total PBC (active cases $161{\cdot}3 \pm 22{\cdot}6$, inactive $135{\cdot}8 \pm 20{\cdot}9$ mg. hexose/100 ml.; $t = 2{\cdot}488$, $P = 0{\cdot}025$) and mucoprotein-bound carbohydrate (active $20{\cdot}9 \pm 7{\cdot}0$, inactive $14{\cdot}3 \pm 4{\cdot}6$; $t = 2{\cdot}349$, $P = 0{\cdot}033$). Most patients with motor neurone disease had raised PBC levels, whereas most with myopathy had not; however, the means for these groups did not differ significantly. Generally, patients with raised total PBC showed an increase in mucoprotein- and (26% Na_2SO_4-separated) albumin-bound carbohydrate. Abnormalities were sometimes observed in electrophoretic patterns (free electrophoresis in barbiturate, pH 8·6, I 0·2), more specifically increases in α-globulins in some cases. Except for serial observations in a case of acute motor polyneuritis, no correlation was found between elevation of α_1- or α_2-globulin and clinical activity in the few cases studied. The patient with polyneuritis showed elevated PBC (total and mucoprotein- and albumin-bound) and α-globulins, all of which returned to normal with recovery.

Although the series (*a*) findings suggest some connexion between elevation of serum PBC and tissue breakdown, the elevation in PBC occurring after injury (Keyser, 1952) could not be correlated with nitrogen catabolism: sometimes the peak was passed before the onset of the catabolic phase, and sometimes the elevation in PBC was maintained after urinary nitrogen excretion had become normal. Generally, albumin- and mucoprotein-bound carbohydrate concentrations paralleled total PBC.

In series (*c*) no consistent changes in total PBC and mucoprotein-bound carbohydrate in relation to

the menstrual cycle were found, confirming and extending the conclusion of Shetlar, Foster, Kelly & Everett (1948) in respect of total PBC.

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The Effect on the Clotting Time of Rat Plasma of a Phosphatidase from *Bacillus cereus*. By D. S. Robinson, P. M. Harris and J. C. F. Poole. (*Sir William Dunn School of Pathology, University of Oxford*)

Ethanolamine phosphatide increases the amount of thrombin generated during plasma coagulation and accelerates the clotting of plasma in the presence of Russell's viper venom (Robinson & Poole, 1956; Poole & Robinson, 1956). It does not accelerate the time taken for citrated rat plasma to clot on adding calcium chloride solution (calcium time) unless most of the particulate lipoid has been previously removed by centrifuging (high-speed spun plasma). Before centrifuging, plasma probably contains sufficient ethanolamine phosphatide within lipoid particles to cause maximal shortening of the calcium time (Robinson & Poole, 1956).

In this communication experiments are described in which high-speed spun rat plasma was incubated at 37° with a partially purified phosphatidase C preparation from *Bacillus cereus*. A similar preparation was reported by Chu (1949) to attack both lecithin and 'cephalin'.

The calcium time of the plasma increased steadily during the incubation period. After 3 hr. it was lengthened to between 60 and 90 min. but returned to the control level after adding ethanolamine phosphatide. Following longer periods of incubation the plasma could not be clotted by calcium chloride solution. It was clotted by calcium chloride solution after adding ethanolamine phosphatide, but the coagulation time was $1\frac{1}{2}$ to 2 times that of control plasma samples containing enzyme and added ethanolamine phosphatide.

Chemical studies showed that during the incubation period the plasma phosphatide was progressively broken down. After 3 hr. incubation some 60 % of the plasma phosphatide had been destroyed. After 15 hr. only 10 % remained. Samples of three phosphatides were incubated with the enzyme. All were broken down, lecithin most rapidly, ethanolamine phosphatide somewhat more slowly, and serine phosphatide slowest of all.

Ethanolamine phosphatide forms approximately 1 % of the total phosphatide of high-speed spun rat plasma. It seems probable that part of this may, under normal circumstances, enter the plasma with dietary fat. In shed blood additional ethanolamine phosphatide is probably liberated from the platelets unless precautions are taken to prevent platelet destruction (O'Brien, private communication). Our experiments suggest that, in the absence of ethanolamine phosphatide, the calcium time is very prolonged.

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The Provitamin A Activity of some Possible Intermediates in β-Carotene Metabolism. By S. Fazakerley and J. Glover. (*Department of Biochemistry, University of Liverpool*)

In studies on the conversion of β-carotene to vitamin A, Glover & Redfearn (1954) demonstrated that the β-8′-, β-10′-, and β-12′-apo-carotenals were vitamin A precursors in the rat. Thus, if β-carotene were oxidatively attacked at a terminal double bond, the remaining β-apo-carotenoid could give rise to vitamin A.

Two possibilities for metabolizing these branch-chain compounds present themselves. They could be converted to the acids and (i) suffer β-oxidation since the methyl groups in the α-position to the terminal carboxyl do not appear to inhibit such a process (Weitzel, 1951) or (ii) two 5-carbon units could be split from β-8′-apo-carotenoic acid or one

unit from β-12′-apo-carotenoic acid. These possibilities were examined by comparing the metabolism of the C_{22}-acids, β-14′-apo-carotenoic acid (retinylideneacetic acid) and 15-hydroxy-retinylacetic acid, with that of the C_{25}-acids, β-12′-apo-carotenoic acid (γ-retinylidenetiglic acid) and γ-15-hydroxy retinyltiglic acid, in vitamin A-deficient rats.

A few mg. of each substance was administered to the rats, and 1–2 days later their intestines, livers and kidneys were found to contain vitamin A. The total yields of the latter were approximately 4% for the C_{22}-compounds but 7–21% for the C_{25}-compounds.

In other tests for growth-promoting activity, the latter were comparable to vitamin A; each facilitating good growth at 2 μg./rat/day, whereas the C_{22}-compounds were less active, 10 μg. of the hydroxy compound and more than 40 μg./rat/day of the other being required.

If γ-retinylidenetiglic acid is degraded by β-oxidation to vitamin A, then either 15-hydroxyretinylacetic or retinylideneacetic acid would be likely intermediates, but neither was detected in the animals dosed with the C_{25}-substances. Together, the above results suggest (i) that the latter are metabolized by another enzyme system which can remove the five terminal carbons as a single unit such as tiglyl–Co A known to be formed in animal tissues (Robinson, Bachhawat & Coon, 1956) and (ii) that while β-oxidation can take place when there is no methyl group in the α-position it is probably not the system involved when there is such substitution.

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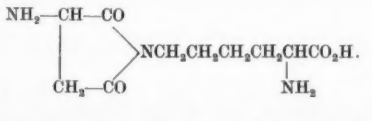
A Cyclic Derivative of Aspartyllysine obtained from Bacitracin A. By D. L. Swallow and E. P. Abraham. (*Sir William Dunn School of Pathology, University of Oxford*)

When bacitracin A was treated with 12N-HCl at 80° most of its constituent amino acids were liberated in the free state, but L-lysine and L-aspartic acid remained bound in a peptide which was surprisingly resistant to acid (Lockhart & Abraham, 1956). In this peptide the amino group of aspartic acid and the α-amino group of lysine were free. However, the peptide had a small net positive charge at pH 7, a fact which suggested that it was not simply an aspartyllysine.

ε-*N*-(α-L-Aspartyl)-L-lysine and ε-*N*-(β-L-aspartyl)-L-lysine have now been synthesized (Swallow, Lockhart & Abraham, to be published). They can be separated by ionophoresis on paper in 10% (v/v) acetic acid. Both peptides show no net charge at pH 7, but on treatment with 12N-HCl at 80° they are converted, in good yield, to a substance which appears to be identical with that obtained from bacitracin and which contains an ionizable group with a pH of about 6·0. It is suggested that cyclization occurs with the formation of an aminosuccinoyllysine:



On treatment with dilute alkali (pH 11), the new compound yields a mixture of α- and β-aspartyllysines. The β-*iso*hexylamide of aspartic acid also appears to undergo cyclization in the presence of 12N-HCl.

The presence of the cyclic peptide in acid hydrolysates of bacitracin A accounts for the difficulty with which the lysine and one of the two aspartic acid residues of the antibiotic are liberated in the free state. It does not follow that a structure of this type occurs in bacitracin A itself, since the conditions used for hydrolysis are suitable for cyclization; also, if bacitracin contains an aspartic acid residue linked to lysine by only one bond, it cannot be decided, from these experiments, whether the linkage involves an α- or a β-carboxyl group.

In addition to 'Asp.Lys', the peptide 'Asp (Ileu).Lys', is formed when bacitracin A is treated with 12N-HCl. This peptide also appears to be a derivative of aminosuccinimide, since it has a small positive charge at pH 7.

Derivatives of aminosuccinimide do not appear to have been previously identified in hydrolysates of polypeptides, but a substance composed largely of aspartic acid and lysine which is resistant to 6N-HCl at 100° has recently been detected among the degradation products of cell walls of lactobacilli (Cummins & Harris, 1956).

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Specific Absorption with Line Extinction in Ouchterlony Plates. By J. G. FEINBERG. (*C. L. Bencard Allergy Research Unit, Beecham Research Laboratories, Betchworth, Surrey*)

The meaning of patterns of precipitation obtained in antigen-antibody systems in Ouchterlony plates is sometimes unclear. The so-called 'reaction of identity', 'reaction of non-identity' and 'reaction of partial identity' have been disputed by a number of investigators (Kaminski, 1954; Jennings, 1954, 1956; Wilson & Pringle, 1954; Korngold, 1956).

A means of eliminating specific lines in agar gels might well prove as important as methods for their formation in furthering an understanding of the phenomena involved. A method has been investigated in this laboratory whereby precipitation lines can be specifically prevented from forming by incorporating an antigen or antiserum in the supporting agar.

The method will be described and slides shown demonstrating its application to two specific systems: ovalbumin-antiovalbumin and fibrinogen-antifibrinogen. In each case it has been possible to eliminate one or more strong specific lines by the principle of specific absorption in the supporting agar. Subsequently it was learnt that Björklund (1953) had described a similar method, so the present report must be considered purely confirmatory,

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Ouchterlony Gel Plate Precipitates with a Simple Chemical Substance: Dihydroxy-di-(*p*-azophenylarsonic acid)-benzene. By J. G. FEINBERG and R. A. GRANT. (*C. L. Bencard Allergy Research Unit, Beecham Research Laboratories, Betchworth, Surrey*)

Investigation of the underlying phenomena involved in antigen-antibody precipitation reactions in Ouchterlony gel plates has hitherto been complicated by the lack of a simple precipitation system. The macromolecular antigens—proteins and polysaccharides—used in gel precipitation are too complex to permit precise definition and give rise to uncertainty of interpretation when seemingly anomalous patterns are obtained.

In the belief that a simple, chemically defined 'antigen' of definite molecular weight and structure would assist in the study and interpretation of gel diffusion phenomena, the authors studied the behaviour in gel plates of an arsanilic acid–resorcinol divalent hapten of the type first used by Landsteiner & van der Scheer (1932*a*, *b*; 1933) and subsequently by Pauling, Pressman, Campbell, Ikeda & Ikawa (1942) for the study of precipitation in tubes. The compound studied, dihydroxy-di-(*p*-azophenylarsonic acid)-benzene, was synthesized by coupling two molecules of diazotized arsanilic acid to one molecule of resorcinol.

Specific antisera were made by immunizing rabbits with azoprotein prepared by coupling diazotized arsanilic acid to 4 times re-crystallized ovalbumin. Specific precipitates were obtained between the divalent hapten and the antisera in Ouchterlony plates. These will be demonstrated and discussed.

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A Non-enzymic Transphosphorylation Catalysed by Manganous Ions. By J. M. LOWENSTEIN.† (*Department of Biochemistry, University of Oxford*)

Studies on the mechanism of enzyme catalysis have stimulated interest in finding analogous non-enzymic reactions. This communication describes a non-enzymic transphosphorylation from adenosine triphosphate (APPP) to orthophosphate (P) which is catalysed by Mn^{2+} ions. The product of the reaction is shown to be inorganic pyrophosphate (PP).

A mixture of 0·5 ml. 0·1M APPP, 0·1 ml. 0·05M-$K_2H^{32}PO_4$ (P*), 0·1 ml. 0·5M-$MnCl_2$, 0·1 ml. 1·0M tris-hydroxymethylaminomethane-HCl buffer, pH 8·0, and 0·2 ml. water was incubated at 25° for 6 hr. At the conclusion of the reaction the ortho-

† Beit Memorial Fellow.

phosphate in the reaction mixture was converted to phosphomolybdate and extracted with *iso*butanol by an adaptation of the method of Berenblum & Chain (1938). The extraction with *iso*butanol was repeated three times, and the aqueous layer was assayed for radioactivity. Starting with 500 000 counts/min. of P*, the radioactivity not extracted by *iso*butanol under the above conditions rose from 0 counts/min. at zero time to 30 000 counts/min. after 6 hr. With $MnCl_2$, APPP, or buffer omitted it rose from 0 to 330, 430, and 9400 counts/min. respectively.

Another portion of the reaction mixture was chromatographed on Dowex-2 resin, 10% cross-linked, 200–400 mesh, formate form, with a resin bed 2·5 cm. high and 1 cm. diameter, using ammonium formate and formic acid as eluents. A radioactive peak corresponding to the position of elution of PP, and containing no material absorbing light at 260 mμ., was present after incubating for 6 hr., but not at zero time. The specific activity of the presumed PP* peak was determined, and was found to be one-half to one-third of the specific activity of P*, the starting material.

The peak presumed to contain PP* was co-chromatographed with authentic PP. The specific activities of each tube of the PP peak were determined, and were found to be identical. This confirmed that the reaction product is PP.

The above data constitute evidence for the reaction $APPP + P^* \xrightarrow{Mn^{2+}} APP + PP^*$. The implications of these findings will be discussed.

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DEMONSTRATIONS

An Electrical Analogue for the Analysis of Multiple Exponential Curves. By L. Bernstein and M. Weatherall. (*Departments of Physiology and Pharmacology, London Hospital Medical College, London, E.* 1)

When a quantity of material is distributed from one compartment into others, or when it decomposes in more than one stage, it is commonly difficult to measure directly the rate constants for the component movements or reactions. The overall movement or reaction velocity can usually be measured, and component velocities are then estimated by trial-and-error methods, which are laborious and time-consuming. When the individual processes or reactions are known to be exponentially related to time a simple solution becomes possible by allowing each process to be represented by the charge or discharge of an electrical condenser through a resistor, a process which also occurs exponentially. A number of condensers and resistors can be connected together with a geometry analogous to the compartments and boundaries of the original system; the condenser capacities can be chosen to be proportional to the compartment sizes and the resistors can be varied until the distribution of charge over the system follows a time course parallel to that in the original system. The resistance values are then inversely related to the corresponding movement rates or reaction velocities in the original system. The distribution of charge can be visualized with the aid of an oscilloscope. The apparatus to be demonstrated enables these operations to be performed with certain refinements, and it will be shown with particular reference to the analysis of cation movements in erythrocytes.

Perfusion of the Lumen of Rat Small Intestine. By M. W. Neil. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

There are many different techniques available for studying the absorption of substances from the small intestine of small laboratory animals. The two techniques demonstrated are minor modifications of standard procedures for perfusing the lumen of a segment of gut (*a*) *in situ*, with the blood circulation intact or (*b*) using an isolated segment bathed on the mucosal and serosal sides with physiological saline. These methods are in use in this laboratory for the study of the absorption of certain amino acids from the rat small intestine.

Haemoglobin Types in British Breeds of Sheep. By J. V. Evans, H. Harris and F. L. Warren. (*Rowett Research Institute, Bucksburn, Aberdeenshire, and Department of Biochemistry, London Hospital Medical College, London, E.* 1)

Two kinds of adult haemoglobin may occur in sheep. They can be readily separated by paper electrophoresis (Harris & Warren, 1955). The kind which migrates more rapidly to the anode at pH 8·6 has been called sheep haemoglobin A, and the less rapidly moving kind sheep haemoglobin B. Three distinct kinds of animal may be distinguished according to whether they possess one, or the other, or a mixture of these two types of haemoglobin. They are conveniently designated as A, B, or AB sheep. The haemoglobin types are inherited and appear to be determined by two allelic genes each responsible for the formation of one kind of haemoglobin (Evans, King, Cohen, Harris & Warren, 1956).

The haemoglobin types in some 4500 sheep representing thirty-three different British breeds have been determined. Large differences in the relative frequencies of the three types of animal occur from breed to breed. For example, in the Scottish Blackface 61 % of the animals were of type A, 33 % were AB, and 6 % were B; in the North Country Cheviots 18 % were A, 51 % were AB, and 31 % were B; in the South Devon 8 % were A, 36 % were AB, and 56 % were B; in the English Leicester no type A animals were observed, 2 % were AB, and 98 % were B. The gene frequencies for the A gene in these breeds were respectively 0·77, 0·43, 0·26, and 0·01. Gene frequencies in different flocks of the same breed were in good agreement.

The significance of this haemoglobin polymorphism in sheep, and the reason for the very large differences in gene frequencies from breed to breed remains obscure. In general, the lowland breeds are predominantly of haemoglobin type B, while haemoglobin A is very much more conspicuous in the mountain and hill breeds. Thus among eighteen lowland breeds, three-quarters of them had gene frequencies for B greater than 0·8, while in the mountain and hill breeds the opposite was the case. This suggests that the haemoglobin type may perhaps be of some adaptive significance. However, so far no obvious differences in any other characteristic of sheep of different haemoglobin types within the same breed have been detected.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **359th Meeting** *of the Biochemical Society was held in the Beveridge Hall of the Senate House, University of London, Malet Street, London, W.C.* 1, *on Saturday,* 23 *February* 1957, *at* 11 *a.m., and took the form of a Symposium on*

STRUCTURE AND FUNCTION OF SUBCELLULAR COMPONENTS

The Ultrastructure of Cell Membranes and their Derivatives. By J. D. ROBERTSON. (*Department of Anatomy, University College, Gower Street, London, W.C.* 1)

A review of certain aspects of the organization of cells at the ultrastructural level will be presented. Particular emphasis will be placed on the cell membrane and its derivatives. Modern concepts of the molecular structure of cell membranes derived from studies of nerve myelin will be considered. The relationship of the cell membrane and nuclear membrane to the endoplasmic reticulum will be treated in a semi-speculative manner and certain current hypotheses regarding the transport of fluids into and through cells will be discussed. The ultrastructure of mitochondria will be discussed in relation to that of cell membranes. Other cytoplasmic constituents will be dealt with briefly.

The relationships which cell membranes assume at interneuronal and neuromuscular junctions will be reviewed and compared with intracellular double membranes and the relationships between epithelial cells. Finally, the special relationships of Schwann cell and axonal membranes in general and at regions of physiological interest, such as nodes of Ranvier and the sensory endings of neuromuscular spindles, will be described.

These regions are characterized not only by special membrane relationships but also by aggregations of mitochondria and vesicular-appearing bodies 300–500 Å in diameter. Certain possible biochemical implications of the concentrations of the latter structures in particular will be discussed. Furthermore, a type of granule newly observed in the Schwann cell at nodes of Ranvier will be described. These are smaller than mitochondria and range down to a few hundred Ångström units in diameter. Their appearance suggests a Schwannian secretory product peculiar to the nodes.

Principles and Potentialities of Cytochemical Staining Methods for the Study of Intracellular Enzyme Distributions. By S. J. HOLT. (*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, Mortimer Street, London, W.* 1)

Cytochemical staining methods for studying the distribution of enzymes depend upon the formation of insoluble coloured indicators at the sites of enzymic activity when cells or tissue sections are treated with solutions containing suitable chromogenic substrates. Ideally, the staining pattern produced should not only indicate the qualitative distribution of the enzyme, but also the relative activities in different locations.

Criteria to which staining methods should conform, for these requirements to be met, will be discussed briefly in the light of recent theoretical analyses of a generalized staining process of the type:

$$\text{Substrate} \xrightarrow[\text{reaction}]{\text{enzymic}} \underset{\text{precursor}}{\text{Diffusing stain}} \xrightarrow[\text{reaction}]{\text{'capture'}} \text{Stain.}$$

The development of practical methods which appear to conform closely to the theoretical requirements will be illustrated by reference to a staining procedure for esterases based upon the use of substituted indoxyl acetates as substrates. These are hydrolysed by the enzyme and the indoxyl liberated is then rapidly oxidized to the corresponding indigoid dye, which serves as the indicator of enzymic activity. Systematic studies with this type of process suggest that esterases may be located in cells with a precision of about 0·5 μ.

Provided the stains produced have a suitable physical form, direct comparison of enzymic activities in different locations is potentially feasible through the use of microspectrophotometric procedures. The possibility of identifying enzymes in cells from a consideration of rates of staining *in situ* will also be discussed.

Distribution and Interaction of Enzymes within Animal Cells. By L. ERNSTER. (*Wenner-Gren Institute, University of Stockholm, Sweden*)

Despite the vast body of information to-day available about the intracellular distribution of enzymes, relatively little is known as to the way in which single enzymes and cell-constituents interact to bring about integrated metabolic reaction sequences. A choice of experimental data, illustrating some relevant problems, will be presented in this paper.

One problem is that the percentage distribution of an enzyme is not necessarily an adequate expression for the distribution of its actual activity among the various cell constituents. This will be illustrated by some recent data on the cytoplasmic distribution of *iso*citric dehydrogenase.

Another difficulty arises from the fact that enzymes may behave in different ways under different experimental conditions. A well-known example is the 'latent' mitochondrial adenosine triphosphatase. As indicated by accumulating experimental evidence, this enzyme activity appears to be a modification of the activity of certain enzymes involved in phosphate transfer. Some aspects of this modification and of its possible reversibility will be discussed in relation to the role played by the mitochondrial structure in oxidative phosphorylation.

Structural factors may play a specially important role in certain synthetic processes, by co-ordinating an adequate supply of high-energy intermediates with suitable steady-state levels of reduced and oxidized pyridine nucleotides. As has recently been shown, under those conditions which favour a maximum efficiency of oxidative phosphorylation in mitochondria, there is virtually no release of reduced pyridine nucleotides from the mitochondrial structure. Under such conditions, oxidations occurring in the soluble fraction of the cytoplasm appear to serve as almost exclusive sources of hydrogen for reductive syntheses. The interaction of these oxidations with mitochondrial terminal electron transport pathways, and its bearing on certain synthetic processes, will be discussed in some detail.

The possible role of cytoplasmic structures in regulating enzymic interaction will be illustrated by some *in vivo* experiments, including electron micrographs of normal and hyperthyroid rat-liver sections. The findings support the hypothesis that hormones may exert their action by controlling interactions between enzymes rather than by acting upon individual enzymes.

Structure and Function in Micro-organisms. By P. MITCHELL. (*Zoology Department, Edinburgh University*)

The aim of this paper is to consider the relationship of structure to function in certain subcellular components of relatively primitive or structurally simple cells. We shall centre attention mainly on bacterial plasma-membranes because bacteria represent structurally the simplest group of organisms in as much as they contain few organelles of dimensions above 20 mμ., and are thus particularly well suited for studies of membrane function. Bacteria lack structures corresponding closely to the nuclear membrane, mitochondria or endoplasmic reticulum of metazoa. The bacterial deoxyribonucleic acid is coiled up as granules in direct osmotic contact with the diffusible components of the cytoplasm. The water-soluble cytoplasmic constituents of molecular weight below 1000 (e.g. inorganic salts, amino acids, nucleotides) may account for 20% of the cell dry weight, and are free to exert an osmotic pressure of 20 to 30 atmospheres against the plasma-membrane. The corresponding hydrostatic pressure is borne by a strong, relatively porous cell-wall, about 20 mμ. thick, lying outside the plasma-membrane. In electron micrographs of thin sections of bacteria, much of the cytoplasm appears to consist of granules 10–20 mμ. in diameter. Particular fractions of diameter from 10 mμ. upwards have been isolated from disintegrated bacteria, and some information about their composition and enzyme activity is available. A considerable proportion of the particles obtained from disintegrated bacteria originate not from the cytoplasm but from the plasma-membrane. Although only 5–10 mμ. thick, the plasma-membrane constitutes 10–20% of the bacterial dry weight or protein. It consists essentially of one or two monolayers of lipoprotein, forming an effective barrier to the diffusion of most solutes carrying more than 4 water molecules. The high specificity and 'Michaelis kinetics' of the uptake of certain solutes through this membrane, and the evidence of enzyme analyses of the isolated membrane, lead to the conclusion that enzymes and carriers, including the cytochrome system and associated dehydro-

genases, form an integral part of the plasma-membrane of bacteria. These enzymes and carriers connect the protoplasm of the cell osmotically with the environment. It is probable that they also mediate in the transfer of the precursors of the cell-wall and capsular substances from the internal metabolic pool to their sites of polymerization outside the plasma-membrane. The versatile and metabolically dominant osmotic linkage functions of bacterial membranes probably have their specialized counterparts in the membranes of the subcellular units and cells of higher organisms.

Changes in Cellular Components in Liver Injury. By J. D. JUDAH and K. R. REES. (*Department of Morbid Anatomy, University College Hospital Medical School, London, and Department of Biochemistry, University College, London*)

Since the development of techniques for the separation of subcellular components (mitochondria, nuclei, and microsomes) numerous investigations have been carried out on changes in their properties and amounts in a variety of experimental conditions.

We propose to discuss acute liver injury produced by carbon tetrachloride (CCl_4), uncomplicated copper deficiency in rats and acute thioacetamide poisoning. In addition, azo dyes, acetylaminofluorene and chronic thioacetamide poisoning which have produced liver tumours will be considered.

In CCl_4 poisoning the primary lesion is thought to be mitochondrial; evidence for this will be presented. In copper deficiency, two main biochemical changes have been found, one a serious depletion of cytochrome oxidase (predominately a mitochondrial enzyme) and the second a failure in phospholipid synthesis. The interrelations and consequences of these will be considered. Acute thioacetamide poisoning produces liver necrosis which has peculiar features; changes in the electrolyte pattern of the liver cells occur, notably a large increase in calcium. When the effect of calcium is counteracted *in vitro* by the addition of ethylenediaminetetra-acetic acid normal levels of respiration are found. Antihistamines prevent the effects of thioacetamide in the intact animal.

In chronic liver injury induced by carcinogens, changes occur in the amounts of some of the subcellular components; qualitative changes may also be seen which differ with the agent used. Examples of these will be given.

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Sorbitol-C14 (G)
Taurine-S35
Glycerol-1-C14
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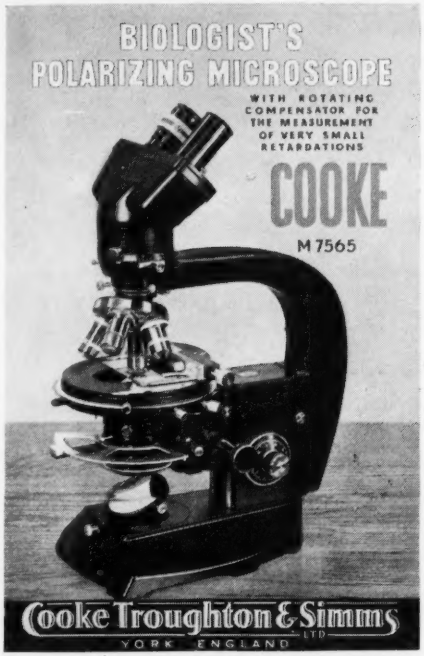

Laccerol, Lactalbumin, Lactalbumin, Lactic acid l, Lactic acid allyl est drogenase, Laetide, La cryst., Lac tophenin, L Lanadigin, Lanatoside terol, Lanthanum, Lanthanum acetate, ferricyanide, Lanthanum formate, Lant Lanthanum, Lauric ester, La ster, Lau Laurovlchloride, Laurovlnero late, n-Lauryl alcohol, Lauryl butyra Lau ethyl cit thyl iodat , L phthalate, Lead potassium iodide, Le Lead Lea tetr e, Le chlo in, Lepidine, Leucine aminopeptidase, Le thionine, Leucoriboflavine, Leucoros Levoglucosan, Levulin ignin benzyl ether, Lignin , Li Linoleic acid, Linol Linol Lipocaic, Lipositol, , Lis Lithi acetylide, l yl ph Lith aluminum hyd ium am hyd lithium ca num ca di Lithium Lithiu

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